Remarks

Applicants appreciate the Examiner's withdrawal of the objections to the specification and claims regarding the use of brackets, the previous rejection of claims 2-16 under 35 U.S.C. § 112, second paragraph, the rejection of claim 2 under 35 U.S.C. § 102(b), and the allowance of claims 14 and 15.

Amendments

Claim 1 has been amended to recite "wherein the chimeric protein binds and inhibits factor VIIa / tissue factor complex and binds to and inhibits factor Xa." This recitation is supported on page 1, lines 4-5 of the specification: "This invention relates to chimeric proteins capable of simultaneously binding and inhibiting factor VIIa/tissue factor complex (factor VIIa/TF complex) and factor Xa."

This amendment was not previously made because Applicants believed their previous response was sufficient to overcome the previous rejections. The amendment adds no new matter and does not require a new search.

Objections to Claims 2 and 16

Claim 2 is objected to because it recites "wherein A, B, C, D, E, F, G may comprise portions of native TFPI or TFPI-2 sequences" and the recitation "or non-native sequence" present in the original claim was not shown as deleted. Applicants thank the Examiner for pointing out the missing recitation, which Applicants did not intend to delete at that time. In the present response, however, the entire recitation "wherein A, B, C, D, E, F, G may comprise portions of native TFPI or TFPI-2 sequences or non-native sequence," is deleted. For clarity, the

entire recitation is shown as deleted in the presently amended version of claim 2.

The Final Office Action objects to the use of brackets in the formula recited in claim 16. To advance prosecution, the formula has been amended to use parentheses instead of brackets.

The Obviousness-Type Double Patenting Rejections

Claims 1-11, 16-25, and 73 stand rejected as obvious over claims 1-17 of U.S. Patent 6,174,721. Claims 1-11, 16-27, and 73 stand rejected as obvious over claims 1-24 of U.S. Patent 5,589,359.

As noted in the Final Office Action, upon an indication of allowability of the pending claims but for the issue of obviousness-type double patenting, Applicants will consider filing a Terminal Disclaimer.

The Rejection of Claims 1-11, 13, 16-27, 73, and 88 Under 35 U.S.C. § 112, first paragraph

Claims 1-11, 13, 16-27, 73, and 88 stand rejected under 35 U.S.C. § 112, first paragraph, as not enabled for the full scope of the recited muteins. Applicants respectfully traverse the rejection.

To satisfy the enablement requirement of 35 U.S.C. § 112, first paragraph, the present specification must teach one of skill in the relevant art how to make and use chimeric proteins comprising a mutein of a Kunitz-type domain 1 or 2 of TFPI or TFPI-2 without the need for undue experimentation. *In re Wright*, 999 F.2d 1557, 1561, 27 U.S.P.Q.2d (BNA) 1510, 1513 (Fed. Cir. 1993). The scope of enablement in the specification must bear a reasonable

correlation to the scope of the recited genera of muteins. *In re Fisher*, 427 F.2d 833, 839, 166 U.S.P.Q. (BNA) 18, 24 (C.C.P.A. 1970). The present specification meets this standard.

The specification defines "mutein" as "a normal or wild-type sequence in which 1-5 amino acid substitutions have been made." Page 7, lines 26-27. In the previous response, Applicants pointed out that the specification teaches numerous examples of such muteins of TFPI and TFPI-2. See page 13, line 17, through page 14, last full paragraph of the response filed February 11, 2003. Applicants also pointed out that the specification teaches how to make the disclosed muteins. See the paragraph bridging pages 14 and 15 of the response filed February 11, 2003. Applicants explained that these teachings bear a reasonable correlation to the scope of the recited genera of muteins of Kunitz-type domains 1 and 2 of TFPI or TFPI-2 and that, provided with these teachings, one skilled in the art would be able to make and use chimeric proteins comprising muteins of Kunitz-type domains 1 or 2 of TFPI or TFPI-2 without undue experimentation. See page 15, first full paragraph of the response filed February 11, 2003.

The Final Office Action asserts that Applicants' arguments were not persuasive "because the mutein cited in the claim does not have any structural or function [sic; functional] description, thus, the claim would include various muteins, not just those indicated in the specification, thus it requires further guidance on the identities of the muteins and further experimentation to assess the inhibitory effects of the chimeric proteins containing these muteins against Factor VIIa/TF/Xa." Page 10, lines 8-12.

To advance prosecution, claim 1 has been amended to clarify that the chimeric protein "binds and inhibits factor VIIa / tissue factor complex and binds to and inhibits factor Xa." This amendment excludes muteins that would render the claimed chimeric proteins non-functional.

Those skilled in the art would readily be able to identify muteins of the recited Kunitz-type domains that fall within the scope of amended claim 2. Methods by which the inhibition of factor VIIa / tissue factor complex and of factor Xa by either TFPI or TFPI-2 can be assayed were known in the art before the priority date of this application. *See, e.g.*, Hamamoto *et al.*, *J. Biol. Chem. 268*, 8704-10, 1993 (attachment 1); Huang *et al.*, "Kinetics of factor Xa inhibition by tissue factor pathway inhibitor," *J. Biol. Chem. 268*, 26950-55, 1993 (attachment 2); Sprecher *et al.*, *Proc. Natl. Acad. Sci. USA 91*, 3353-57, 1994 (attachment 3, referred to in the specification at page 2, line 11). Such methods could easily be performed to determine whether a chimeric protein comprising any particular mutein(s) of the recited Kunitz-type domains had the recited functional properties. Because the specification provides guidance as to how to make such muteins and because the art knew how to assay TFPI and TFPI-2 proteins for the recited functions, such screening would merely have been routine.

Applicants respectfully request withdrawal of the rejection.

The Rejection of Claims 2-13 Under 35 U.S.C. § 112, second paragraph

Claims 2-13 stand rejected under 35 U.S.C. § 112, second paragraph, as indefinite. Applicants respectfully traverse the rejection.

The rejection is based in part on the asserted indefiniteness of the recitation "wherein a, b are integers from 0-6." The Final Office Action asserts that if either a or b is 0, the chimeric protein A would not contain the two Kunitz domains recited in claim 1. The Final Office Action acknowledges that because claims 2-13 depend from claim 1 and because claim 1 recites two

Kunitz domains, the combination a=b=0 is precluded. For the same reason, neither a nor b can be 0.

The Final Office Action also asserts that the recitation "wherein A, B, C, D, E, F, G may comprise portions of native TFPI or TFPI-2 sequences or non-native sequence" is indefinite. To advance prosecution, this recitation has been deleted.

Claims 2-13 are clear and definite as written. Applicants respectfully request withdrawal of the rejection.

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Respectfully submitted,

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Inhibitory Properties of Full-length and Truncated Recombinant Tissue Factor Pathway Inhibitor (TFPI)

EVIDENCE THAT THE THIRD KUNITZ-TYPE DOMAIN OF TFPI IS NOT ESSENTIAL FOR THE INHIBITION OF FACTOR VIIa-TISSUE FACTOR COMPLEXES ON CELL SURFACES*

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Human tissue factor pathway inhibitor (TFPI) is a plasma protease inhibitor that consists of three tandem Kunitz-type inhibitor domains flanked by a negatively charged NH_2 terminus and a positively charged COOH-terminal tail. Previous studies have shown that the first and second Kunitz-type domains in TFPI are involved in the inhibition of factor VIIa and factor Xa activity, respectively. In the present study, we have compared the inhibitory properties of full-length recombinant TFPI and a truncated form of TFPI lacking the third Kunitz-type domain and COOH-terminal tail ($TFPI^{1-161}$) with respect to inhibition of factor VIIa-tissue factor complexes on the surface of a human bladder carcinoma cell line J82. Full-length TFPI and $TFPI^{1-161}$ were kinetically indistinguishable with respect to neutralization of the proteolytic activity of preformed complexes of factor VIIa-tissue factor on the J82 cell surface in the absence of factor Xa. Equimolar amounts of factor Xa augmented the anticoagulant activity of both preparations of TFPI to the same extent, and both preparations of TFPI were equally effective in inhibiting factor VIIa-tissue factor amidolytic activity in solution phase. In addition, plasma concentrations of both forms of TFPI, in stoichiometric complex with factor Xa, inhibited cell surface factor VIIa-tissue factor proteolytic activity markedly faster than plasma levels of antithrombin III, even in the presence of 1 unit/ml heparin. The results of displacement studies suggested slight differences in the affinity of the two TFPI molecules for the cell surface in that ~5% of a VIIa·TF·Xa·$TFPI^{1-161}$ quaternary complex on J82 cells was displaceable from the cell surface by high concentrations of factor VIIa (10–100 nM), whereas only 1–2% of a VIIa·TF·Xa·TFPI complex was displaceable under comparable conditions. Pretreatment of the cells with TFPI/Xa alone or together with R152E factor VII, followed by factor VIIa treatment, revealed significant differences in the two TFPI forms with respect to the degree with which offered factor VIIa could restore factor X activation on the cell surface. These differences notwithstanding, our collective findings indicate that the third Kunitz-type domain and/or COOH-terminal tail of TFPI is not essential for the inhibition of cell surface factor VIIa-tissue factor complexes and suggests that $TFPI^{1-161}$ may be a useful therapeutic agent in the treatment of thromboembolic episodes.

The extrinsic pathway of blood coagulation is initiated when circulating factor VII or factor VIIa binds to its cofactor, tissue factor, presented by many cell types in extravascular tissues (1). This cell surface factor VIIa-tissue factor complex rapidly activates factor IX and factor X by limited proteolysis, which eventually leads to the formation of thrombin and a fibrin clot. Tissue factor pathway inhibitor (TFPI)[1] is a circulating multidomain Kunitz-type protease inhibitor that is thought to play a major role in the regulation of factor VIIa-tissue factor proteolytic activity *in vivo* (for reviews, see Refs. 2 and 3). TFPI is a 42-kDa glycoprotein that consists of a negatively charged NH_2-terminus, three tandem Kunitz-type inhibitor domains, and a positively charged COOH-terminal tail (4). The majority of plasma TFPI circulates in complex with plasma lipoproteins (5, 6), although the functional significance of this complex has yet to be elucidated. Preliminary evidence suggests that the COOH-terminal tail of TFPI plays a role in the association of TFPI with lipoproteins (7). Although the integrity of the TFPI COOH-terminal tail does not appear to be important for its activity in a two-stage chromogenic assay, an intact undegraded COOH-terminal tail in TFPI is essential for maximum expression of its anticoagulant activity in a dilute tissue factor-based coagulation assay (8, 9).

Based on the results of several studies (10–12), TFPI appears to inhibit factor VIIa-tissue factor by a novel two-step reaction mechanism. In the first step, factor Xa binds to the second Kunitz-type domain of TFPI in a calcium-independent manner, resulting in the loss of factor Xa proteolytic and amidolytic activity. In a subsequent calcium-dependent step,

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[1] The abbreviations used are: TFPI, full-length tissue factor pathway inhibitor; $TFPI^{1-161}$, recombinant COOH-terminal truncated TFPI containing residues 1–161; EGRck, Glu-Gly-Arg-chloromethyl ketone; DEGRck, dansyl-Glu-Gly-Arg-chloromethyl ketone; GD, Gla-domainless; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl.

the TFPI/Xa complex interacts with and neutralizes factor VIIa through an interaction of the first Kunitz-type domain of TFPI with the active site of factor VIIa. Whether this two-step mechanism for the inhibition of factor VIIa-tissue factor by TFPI occurs exclusively *in vivo* is unknown. Recent studies indicate that although factor Xa greatly potentiates the TFPI-mediated inactivation of cell surface factor VIIa-tissue factor complexes, TFPI alone significantly inhibited the activation of factor IX by factor VIIa in complex with cell surface tissue factor (13). In addition, earlier work from our laboratory demonstrated that recombinant TFPI inhibited the amidolytic activity of solution-phase factor VIIa-tissue factor in a dose-dependent and factor Xa-independent manner (14).

The function, if any, of the third Kunitz-type domain of TFPI remains unknown. In an effort to elucidate a role for this domain in TFPI activity, we have compared the ability of full-length TFPI with that of a COOH-terminal truncated derivative of TFPI, $TFPI^{1-161}$, lacking the third Kunitz-type domain and the COOH-terminal tail, to inhibit the proteolytic activity of factor VIIa in complex with cell surface tissue factor. We report herein the inhibitory properties of these two TFPI preparations, which appear indistinguishable insofar as their ability to inhibit cell surface factor VIIa-tissue factor complexes.

EXPERIMENTAL PROCEDURES

Materials—Bovine serum albumin (fatty acid-free), phosphatidylcholine, phosphatidylserine, trypsin (1 × solution; tissue culture grade), penicillin-streptomycin (10^4 units of penicillin and 10 mg of streptomycin/ml) and Hepes were obtained from Sigma. Sodium heparin (100 USP units/ml) was obtained from Solopak Laboratories. Affi-Gel 10 and Affi-Gel 15 were purchased from Bio-Rad. Benzoyl-Ile-Glu-Gly-Arg-*p*-nitroanilide (S-2222) and H-D-Ile-Pro-Arg-*p*-nitroanilide (S-2288) were obtained from Helena Laboratories. Glu-Gly-Arg-chloromethyl ketone (EGRck) and dansyl-Glu-Gly-Arg-chloromethyl ketone (DEGRck) were obtained from Calbiochem. Tissue culture flasks and 24-well plates were obtained from Corning. Microtitration plates (96 wells) were obtained from Dynatech Laboratories. Minimum essential medium (Eagle's) and nonessential amino acids were purchased from Mediatech. Fetal bovine serum was obtained from HyClone Laboratories. All other reagents were the highest purity commercially available.

Cell Culture—Human bladder carcinoma J82 cells (ATCC HTB-1) were cultured as described previously (15). Factor X activation studies on J82 cell monolayers were performed within 24 h after reaching confluence in 24-well plates (2 cm^2/well).

Proteins—Full-length recombinant human TFPI was expressed in baby hamster kidney cells and purified as described (8). A carboxyl-terminal truncated variant of TFPI, $TFPI^{1-161}$, lacking the third Kunitz-type domain and the COOH-terminal tail, was expressed in *Saccharomyces cerevisiae* and purified by a combination of ion exchange and gel permeation column chromatography. The details of the purification and characterization of $TFPI^{1-161}$ will be reported separately.[2] Human plasma-derived factor X, factor Xa, and antithrombin III were purified as described (16, 17). Published procedures were used to purify rabbit anti-TFPI IgG (18), Gla-domainless factor Xa (18), DEGRck-inactivated factor Xa (18), recombinant wild-type factor VIIa (19), recombinant R152E factor VII[3] (20) and recombinant S344A factor VII[3] (21). S344A factor VIIa was prepared by incubating S344A factor VII with factor Xa (E/S = 1/500) at 37 °C for 1 h in the presence of TBS (TBS, 50 mM Tris-HCl (pH 7.5) containing 100 mM NaCl), 5 mM $CaCl_2$, and 10 μM phosphatidylcholine/phosphatidylserine (70/30, mol/mol) followed by purification on a CaFVII22-Affi-Gel 10 column as described (21). The purified S344A factor VIIa (10 μM) was then treated with DEGRck (1 μM) at 37 °C for 1 h to inactivate trace amounts of factor Xa and subsequently dialyzed extensively against TBS. Recombinant human tissue factor apoprotein, produced in *Escherichia coli* and purified by immunoaffinity chromatography (22), was kindly provided by Dr. Gordon Vehar, Genentech, Inc., South San Francisco. Relipidation of tissue factor apoprotein was carried out as described (21). The effective tissue factor apoprotein concentration in relipidated samples was assumed to be 50% of the total tissue factor apoprotein concentration (23). A calcium-dependent murine anti-human factor X monoclonal antibody (mAb CaFX40) was produced in Balb/c mice essentially according to Kohler and Milstein (24) and purified from ascites fluid by affinity chromatography on a human factor X-Affi-Gel 15 column equilibrated at room temperature with TBS, 5 mM $CaCl_2$. The mAb CaFX40 was eluted from the affinity column with TBS, 30 mM EDTA and dialyzed against TBS, 0.02% NaN_3. All proteins were homogeneous as judged by SDS-polyacrylamide gel electrophoresis.

General Methods—SDS-polyacrylamide slab gel electrophoresis was performed according to Laemmli (25) using 10% polyacrylamide separating gels. Standard DNA techniques were carried out as described (26). Synthetic oligonucleotides were prepared by solid-phase phosphoramidite chemistry on an automated synthesizer (Applied Biosystems model 380). Nucleotide sequence determinations were performed by the dideoxy chain termination technique (27). Factor VII concentrations were determined by an enzyme-linked immunosorbent assay (28) essentially as described (20). The concentrations of all other proteins used in this study were determined according to Bradford (29) using bovine serum albumin as the reference protein.

Preparation and Purification of S376A Factor X—The human factor X cDNA was obtained from Dr. Earl Davie, University of Washington, Seattle. The natural *Eco*RI site encoded within the coding sequence of factor X had previously been silently mutated to result in a factor X cDNA that was entirely contained in a single 1500-nucleotide *Eco*RI fragment. Two independent oligonucleotide-directed mutagenesis protocols (30) were employed to create amino acid substitution mutations. The first was to mutate the active site serine 376 to alanine. The second mutation changed the threonine at position −2 in the propeptide to arginine. RF DNA was prepared for each of these mutants and recombined to create a mutant factor X cDNA that contained both sequence changes. A 1090-nucleotide *Pst*I-*Eco*RI 3′ end fragment containing the S376A mutation was ligated within a 410-base pair 5′ end *Eco*RI-*Pst*I fragment containing the T-2R mutation, and both were ligated into the *Eco*RI site of the mammalian cell vector Zem229R (31). The resulting construct was sequenced to verify the presence of both mutations and the absence of additional mutagenesis-induced changes. Baby hamster kidney cells were transfected with the factor X expression plasmid by the calcium phosphate procedure (32). For selection of stable colonies, the cells were divided 48 h after transfection into medium containing 250 nM methotrexate. Colonies were screened for factor X production by an immunofilter assay (33) and grown individually for protein analysis. One clone was selected for its stability and expression level and initially grown in serum-containing medium and subsequently in serum-free medium containing 5 μg/ml vitamin K. Serum-free culture supernatants were collected, made 50 mM in benzamidine, filtered through a 0.22-μm filter, and subsequently made 10 mM in $CaCl_2$. The calcified filtered medium was then applied to a column (1.6 × 15 cm) of mAb CaFX40-Affi-Gel 10 equilibrated at 4 °C with TBS, 10 mM $CaCl_2$ at a flow rate of ~30 ml/h. Following sample application, the column was washed with TBS, 10 mM $CaCl_2$. S376A factor X was then eluted from the column with TBS, 30 mM EDTA, and stored at −80 °C. Purified S376A factor X was devoid of coagulant activity and exhibited no amidolytic activity following incubation with the factor X activator from Russell's viper venom in the presence of calcium.

Inhibition of Factor VIIa Activity on J82 Cells by Recombinant TFPI—Wells of a 24-well plate were seeded with 5 × 10^5 J82 cells and grown to confluence. Each well was washed once with buffer A (10 mM Hepes (pH 7.45) containing 137 mM NaCl, 4 mM KCl, and 11 mM glucose) supplemented with 10 mM EDTA and subsequently washed three times with buffer A. Each well was then incubated with 10 nM recombinant factor VIIa in buffer A supplemented with 0.5% bovine serum albumin and 5 mM $CaCl_2$ (buffer A+) for 2 h at 37 °C with constant oscillation (50 rpm) on an orbit shaker (Lab-Line). After this period, cells were washed six times with buffer A+. TFPI (2.5 nM), or a preformed complex of TFPI/Xa (2.5 nM TFPI/2.5 nM factor Xa), dissolved in buffer A+, were added to the cells in a volume of 250 μl and incubated at 37 °C. At selected times, the supernatants were aspirated and each well washed six times with buffer A+. Factor X activation on the washed cell surface was then determined in a

[2] Petersen, J. G., Meyn, G., Rasmussen, J. S., Petersen, J., Jonassen, I., Christiansen, L., and Nordfang, O. (1993) *J. Biol. Chem.* **268**, in press.

[3] Mutant factor VII and factor X are designated according to the notation described by Shapiro and Vallee (39), in which the single-letter code for the original amino acid is followed by its position in the sequence and the single-letter code for the new amino acid.

two-stage assay using the factor Xa-specific chromogenic substrate S-2222. In this assay, each well was treated with 500 μl of buffer A+ containing 267 nM factor X. After 5 min at room temperature, an aliquot (250 μl) of the supernatant was transferred to a tube containing 10 μl of 0.5 M EDTA. An aliquot (100 μl) of this incubation mixture was added to a polystyrene cuvette containing 900 μl of 50 mM Tris-HCl (pH 8.3), 150 mM NaCl, 110 μM S-2222. The absorbance at 405 nm was continuously recorded using a Beckman DU-65 spectrophotometer. Factor Xa concentrations were interpolated from a standard curve relating ΔA_{405}/min and factor Xa concentrations. In some experiments, TFPI/Xa complexes were substituted with a mixture of TFPI (2.5 nM) and either plasma-derived factor X (100 nM) or S376A factor X (100 nM). In addition, the ability of antithrombin III (3 μM), in the presence or absence of factor X (100 nM) or heparin (1 unit/ml), to inhibit the activation of factor X by cell surface factor VIIa-tissue factor was also assessed in this system.

Inhibition of Factor VIIa-Tissue Factor Amidolytic Activity by TFPI—Relipidated recombinant human tissue factor apoprotein (20 nM effective concentration) and factor VIIa (20 nM) were coincubated in a 96-well microtitration plate in 50 μl of buffer A+. Following a 5-min incubation at 37 °C, 50 μl of either TFPI (20 nM), TFPI/Xa (20 nM/20 nM), or TFPI/factor X (20 nM/100 nM) was added to the well and incubated at 37 °C. At selected times, 40 μl of S-2288 (final concentration, 0.57 mM) was added to the well and the absorbance at 405 nm determined continuously in a kinetic microplate reader (Molecular Devices model UVmax).

Effect of TFPI and TFPI/Xa on the Displacement of S344A Factor VIIa from Cell Surface Tissue Factor by Factor VIIa—Confluent monolayers of J82 cells in 24-well plates were initially washed as described above. The J82 cell monolayers were then incubated for 2 h at 37 °C with 250 μl of buffer A+ containing 10 nM S344A factor VIIa in the presence or absence of TFPI/Xa (2.5 nM/2.5 nM) or TFPI/GD-Xa (2.5 nM/2.5 nM). Following this incubation, wells were washed six times with buffer A+ and subsequently treated with various concentrations of factor VIIa (0–100 nM) in 250 μl of buffer A+. After an additional incubation for 2 h at 37 °C, wells were washed six times with buffer A+ and factor X activation on the cell surface determined as described above.

RESULTS

Inhibition of Factor VIIa-Tissue Factor Activity on J82 Cells by TFPI—The ability of plasma concentrations (2.5 nM) of TFPI and $TFPI^{1-161}$ to inhibit the proteolytic activity of factor VIIa-tissue factor complexes on J82 monolayers were compared in the presence and absence of factor Xa, GD-factor Xa, DEGRck-factor Xa, factor X, and recombinant S376A factor X. In these experiments, J82 cell surface tissue factor was initially saturated with factor VIIa. TFPI, or an equimolar complex of TFPI/Xa preincubated for 30 min at 37 °C, was then added to the cell surface and incubated for varying times. At each incubation time point, the cells were washed and residual factor VIIa-tissue factor activity assessed as judged by its ability to activate factor X. In those experiments using factor X, the TFPI and factor X (or S376A factor X) were added simultaneously without a preincubation step. Fig. 1 illustrates that both preparations of TFPI inhibited cell surface factor VIIa-tissue factor activity ~20% in a 60-min incubation period in the absence of factor X or factor Xa. These results are in good agreement with that of Callander *et al.* (13) who first reported the inhibition of the factor VIIa-mediated activation of factor IX by 2.7 nM TFPI on an ovarian carcinoma cell line that constitutively expresses cell surface tissue factor. In the presence of factor Xa, factor X, and S376A factor X, both TFPI and $TFPI^{1-161}$ were kinetically indistinguishable with respect to factor VIIa-tissue factor neutralization rate (Fig. 1). Interestingly, S376A factor Xa, generated in the initial phase of the incubation, augmented the activity of both TFPI preparations to the same extent as factor Xa and revealed that the active site serine residue in factor Xa was not essential for its interaction with TFPI (Fig. 1). Consistent with earlier results (10), neither GD-factor Xa nor DEGRck-factor Xa were able to potentiate the activity of

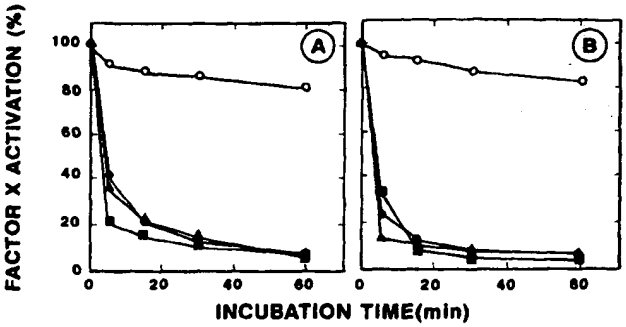

FIG. 1. **Inhibition of factor VIIa-tissue factor activity by TFPI on J82 cells: effect of factor Xa and factor X.** Confluent monolayers of J82 cells in 24-well plates were preincubated for 2 h at 37 °C with 10 nM factor VIIa, washed six times with buffer A+, and subsequently incubated for varying times with either 2.5 nM TFPI (○), 2.5 nM TFPI, 2.5 nM factor Xa (●), 2.5 nM TFPI, 100 nM factor X (▲), or 2.5 nM TFPI, 100 nM S376A factor X (■). Following incubation, cells were washed six times with buffer A+ and factor X activation assessed on the cell surface as described under "Experimental Procedures." *A*, TFPI; *B*, $TFPI^{1-161}$.

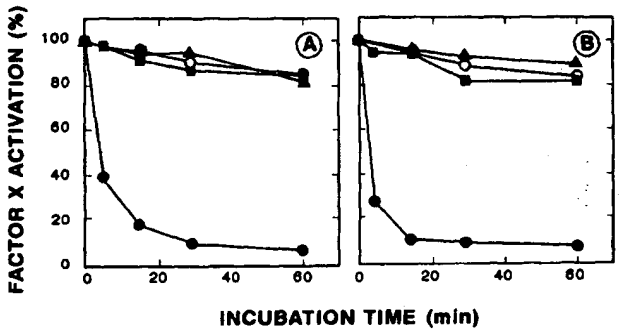

FIG. 2. **Inhibition of factor VIIa-tissue factor activity by TFPI on J82 cells: effect of factor Xa, GD-factor Xa, and DEGRck-inactivated factor Xa.** Confluent monolayers of J82 cells in 24-well plates were preincubated for 2 h at 37 °C with 10 nM factor VIIa, washed, and subsequently incubated for varying tines at 37 °C with either 2.5 nM TFPI (○), 2.5 nM TFPI, 2.5 nM factor Xa (●), 2.5 nM TFPI, 2.5 nM GD-factor Xa (▲), or 2.5 nM TFPI/2.5 nM DEGRck-factor Xa (■). Following incubation, cell were washed six times with buffer A+ and factor X activation assessed on the cell surface as described under "Experimental Procedures." *A*, TFPI; *B*, $TFPI^{1-161}$.

either TFPI preparation on the cell surface (Fig. 2). In light of the fact that S376A factor Xa augmented TFPI activity while DEGRck-factor Xa failed to do so, it is reasonable to conclude that the bulky DEGRck alkylating agent sterically blocks the factor Xa active site pocket, thus preventing the interaction of the second Kunitz-type domain in TFPI with the factor Xa active site. Presumably, the inability of diisopropylphosphoryl-factor Xa to form a complex with TFPI (10) also relates to a bulky substituent in the factor Xa active site pocket.

Inhibition of Factor VIIa-Tissue Factor Amidolytic Activity by TFPI—Earlier work from our laboratory indicated that TFPI inhibited the amidolytic activity of a factor VIIa-tissue factor complex in the absence of factor X or factor Xa (14). Accordingly, we examined whether or not TFPI and $TFPI^{1-161}$ were equally effective in inhibiting factor VIIa-tissue factor amidolytic activity toward the chromogenic substrate, S-2288, in the presence and absence of added factor Xa or factor X. Fig. 3 demonstrates that TFPI and $TFPI^{1-161}$ both inhibited factor VIIa-tissue factor amidolytic activity at essentially the same rate in the absence of factors X and Xa, confirming our earlier finding with TFPI (14). In the presence of factor Xa

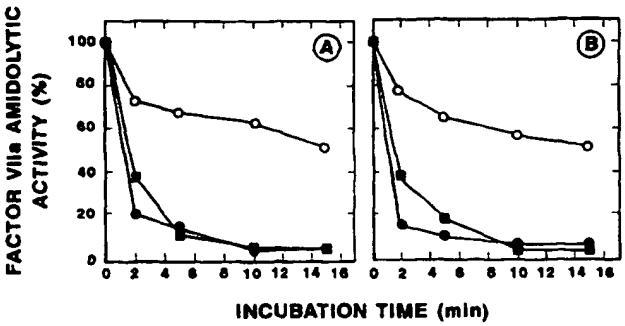

FIG. 3. **Inhibition of factor VIIa-tissue factor amidolytic activity by TFPI: effect of factor Xa and factor X.** Preformed complexes of relipidated tissue factor apoprotein (10 nM effective concentration) and 10 nM factor VIIa were incubated for varying times at 37 °C with either 10 nM TFPI (○), 10 nM TFPI, 10 nM factor Xa (●), or 10 nM TFPI, 100 nM S376A factor X (■). Following incubation, the amidolytic activity of factor VIIa-tissue factor was determined in a kinetic microplate reader using S-2288 as the chromogenic substrate. *A*, TFPI; *B*, $TFPI^{1-161}$.

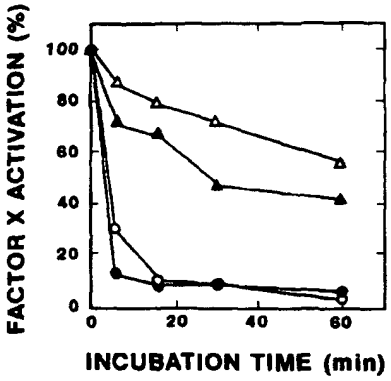

FIG. 4. **Inhibition of factor VIIa-tissue factor activity on J82 cells by TFPI/Xa and antithrombin III: effect of heparin.** Confluent monolayers of J82 cells in 24-well plates were preincubated for 2 h at 37 °C with 10 nM factor VIIa, washed six times with buffer A+, and subsequently incubated for varying times at 37 °C with either 2.5 nM TFPI, 2.5 nM factor Xa (○, ●) or 3 μM antithrombin III (△, ▲) in the presence (●, ▲) or absence (○, △) of 1 unit/ml heparin. Following incubation, the cells were washed six times with buffer A+ and factor X activation assessed on the cell surface as described under "Experimental Procedures."

or S376A factor X, both preparations of TFPI rapidly inhibited factor VIIa-tissue factor amidolytic activity at virtually the same rate (Fig. 3).

Inhibition of Factor VIIa-Tissue Factor Activity on J82 Cells by TFPI/Xa and Antithrombin III—The preceding studies demonstrated that plasma concentrations of TFPI, in the presence of factor Xa or factor X, rapidly neutralized factor VIIa-tissue factor proteolytic activity on cell surfaces. Inasmuch as the plasma concentration of antithrombin III is essentially 3 orders of magnitude higher than TFPI and conceivably could contribute to the neutralization of factor VIIa-tissue factor activity *in vivo*, we next designed experiments to assess the relative abilities of plasma concentrations of TFPI/Xa complexes and antithrombin III, in the presence or absence of heparin, to neutralize cell surface factor VIIa-tissue factor proteolytic activity. In the absence of heparin, plasma concentrations of antithrombin III (3 μM) inhibited cell surface factor VIIa-tissue factor activity at a relatively slow rate (Fig. 4). In the presence of 1 unit/ml heparin, the ability of antithrombin III to neutralize factor VIIa-tissue factor was modestly enhanced such that ~50% of cell surface factor VIIa-tissue factor activity was neutralized in 30 min. In comparison, plasma levels of TFPI/Xa (2.5 nM each) rapidly inhibited cell surface factor VIIa-tissue factor such that factor VIIa activity was inhibited 50% in ~4 min and >90% inhibited in ~15 min. In contrast to antithrombin III activity, heparin (1 unit/ml) did not measurably enhance the rate of TFPI/Xa neutralization of factor VIIa-tissue factor activity (Fig. 4). $TFPI^{1-161}$/Xa (2.5 nM each), in the presence and absence of heparin, produced essentially the same rate of factor VIIa-tissue factor inhibition as the same concentration of TFPI/Xa (data not shown).

In another series of experiments, we investigated the effects of various combinations of TFPI (2.5 nM), antithrombin III (3 μM), heparin (1 unit/ml), and factor X (100 nM) with respect to inhibition of cell surface factor VIIa-tissue factor after a 30-min incubation at 37 °C. The results of these studies are presented in Table I. Interestingly, the copresence of antithrombin III, heparin, and factor X resulted in ~85% inhibition of factor VIIa-tissue factor and was essentially identical to that observed with the copresence of TFPI, antithrombin III, and factor X. Deletion of heparin from this system produced a factor X activation rate essentially identical to that observed for antithrombin III alone (69 *versus* 70%). Furthermore, decreasing the heparin concentration in the system increased the rate of factor X activation in a dose-dependent manner. For example, factor X activation rates of 32 and 42% were observed in the presence of 0.1 unit/ml and 0.01 unit/ml heparin, respectively. Both plasma-derived factor X and recombinant S376A factor X, at 100 nM concentration, were equally effective in inhibiting cell surface factor VIIa-tissue factor in the presence of antithrombin III and heparin. In addition, the inhibitory effect of this combination was in all likelihood not due to TFPI contaminating the antithrombin III preparation, as antithrombin III preparations preincubated with either normal rabbit IgG or rabbit anti-TFPI IgG produced identical results (data not shown). Precisely how heparin and factor X modulate the inhibitor activity of antithrombin III in this system is unknown and merits further investigation.

Effect of TFPI and TFPI/Xa on the Displacement of S344A Factor VIIa from Cell Surface Tissue Factor by Factor VIIa—In the next series of experiments, we investigated whether the third Kunitz-type domain and COOH-terminal tail of TFPI were involved in the stabilization of the VIIa·TF·Xa·TFPI quaternary complex on the cell surface. In these exper-

TABLE I

Neutralization of cell surface factor VIIa-tissue factor by various combinations of TFPI, antithrombin III, heparin, and factor X

Additive				Factor X activation rate
TFPI[a]	ATIII[b]	Hep[c]	X[d]	
				nM/min
−	−	−	−	3.00
+	+	+	+	0.21
+	−	+	+	0.30
+	−	−	+	0.36
+	+	−	+	0.42
−	+	+	+	0.42
−	+	+	−	1.44
−	+	−	+	2.07
−	+	−	−	2.10
+	−	−	−	2.28
+	−	+	−	2.40

[a] Full length TFPI (2.5 nM).
[b] Antithrombin III (3 μM).
[c] Heparin (1 unit/ml).
[d] Factor X (100 nM).

iments, S344A factor VIIa (10 nM), TFPI (2.5 nM), and factor Xa (2.5 nM) were initially coincubated on the J82 cell surface for 2 h at 37 °C. Following a series of washes, the cells were offered varying concentrations of factor VIIa and incubated another 2 h at 37 °C. Following another series of washes, the ability of cell surface factor VIIa-tissue factor to activate factor X was determined. The results of these studies are shown in Fig. 5. Pretreatment of the cells with S344A factor VIIa alone, or in combination with a preformed complex of TFPI·GD-Xa, followed by treatment with increasing concentrations of factor VIIa, resulted in displacement of the S344A factor VIIa or the S344A factor VIIa·TFPI·GD-Xa ternary complex, from tissue factor and regeneration of functional factor VIIa-tissue factor sites. Half-maximal factor X activation rate was observed with 3–5 nM offered factor VIIa (Fig. 5). In sharp contrast, pretreatment of the cells with either S344A factor VIIa/TFPI/Xa or S344A factor VIIa/$TFPI^{1-161}$/Xa, followed by treatment with increasing concentrations of factor VIIa, resulted in the regeneration of 1–2 and 5% of potential factor VIIa-tissue factor activity, respectively (Fig. 5). Increasing the TFPI/Xa or $TFPI^{1-161}$/Xa concentrations to 10 nM each completely prevented the subsequent displacement of S344A factor VIIa from cell surface tissue factor (data not shown). Similar results were observed when S344A factor VIIa was replaced with factor VIIa in this system, although factor VIIa·TF·Xa·TFPI displacement was slightly higher at 100 nM offered factor VIIa (5 and 10% for TFPI and $TFPI^{1-161}$, respectively, compared with 2 and 5% using S344A factor VIIa). These findings indicate that the factor VIIa active site serine residue is not essential for complex formation with TFPI/Xa and suggests that VIIa·TF·Xa·TFPI quaternary complexes exhibit a slightly higher affinity for the cell surface than VIIa·TF·Xa·$TFPI^{1-161}$ quaternary complexes.

We next investigated whether a mutant factor VII molecule (R152E factor VII), incapable of activation, could serve to form a quaternary complex of factor VII·TF·Xa·TFPI on the cell surface. To our surprise, coincubation of R152E factor VII with TFPI/Xa resulted in only 30% of maximal factor X activation rate on the cell surface, whereas coincubation of R152E factor VII with $TFPI^{1-161}$/Xa resulted in ~60% maximal factor X activation rates on the cell surface (Fig. 6). SDS-polyacrylamide gel electrophoresis and autoradiography experiments using ^{125}I-labeled R152E factor revealed no change

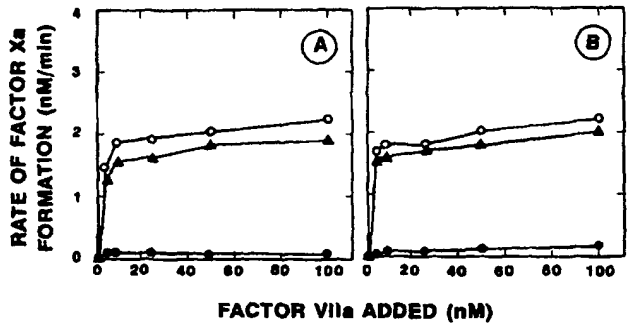

FIG. 5. **Effect of TFPI and TFPI/Xa on the displacement of S344A factor VIIa from cell surface tissue factor by factor VIIa.** Confluent monolayers of J82 cells in 24-well plates were incubated for 2 h at 37 °C with either 10 nM S344A factor VIIa (○), 10 nM S344A factor VIIa, 2.5 nM TFPI, 2.5 nM factor Xa (●), or 10 nM S344A factor VIIa, 2.5 nM TFPI, 2.5 nM GD-factor Xa (▲). Following incubation, cells were washed six times with buffer A+ and treated with various concentrations of factor VIIa. After a 2-h incubation at 37 °C, cells were washed and factor X activation assessed on the cell surface as described under "Experimental Procedures." *A*, TFPI; *B*, $TFPI^{1-161}$.

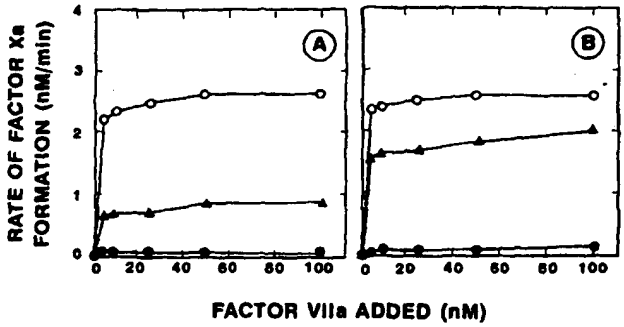

FIG. 6. **Effect of TFPI/Xa on the displacement of R152E factor VII from cell surface tissue factor by factor VIIa.** Confluent monolayers of J82 cells in 24-well plates were incubated for 2 h at 37 °C with either 10 nM R152E factor VII (○) or 10 nM R152E factor VII, 2.5 nM TFPI, 2.5 nM factor Xa (▲). Cells were then washed six times with buffer A+ and subsequently incubated for 2 h at 37 °C with various concentrations of factor VIIa. Following this incubation, cells were washed six times and factor X activation assessed on the cell surface as described under "Experimental Procedures." The effect of coincubating S344A factor VIIa/TFPI/Xa prior to factor VIIa treatment (taken from Fig. 5) is included for comparison (●). *A*, TFPI; *B*, $TFPI^{1-161}$.

in R152E factor VII structure throughout the incubation period (data not shown). This result suggested that zymogen factor VII could participate in the formation of a VII·TF·Xa·TFPI quaternary complex on the cell surface. However, subsequent studies revealed that pretreatment of the cells with TFPI/Xa (2.5 nM/2.5 nM) alone for 2 h at 37 °C, followed by washings and treatment with increasing concentrations of factor VIIa resulted in factor X activation rates identical to that observed if R152E factor VII was included in the first incubation step. Preincubation of the cells with 10 nM TFPI, 10 nM Xa, or 10 nM $TFPI^{1-161}$, 10 nM Xa, further reduced the rate of factor X activation by an additional 20–30% in each case (data not shown). These results suggest that complexes of TFPI/Xa bind to the cell surface in close proximity to the extracellular domain of tissue factor apoprotein and rapidly inactivate factor VIIa once it binds to the tissue factor apoprotein. Presumably, the Gla-domain of factor Xa plays a major role in anchoring this bimolecular complex to acidic phospholipids surrounding the tissue factor apoprotein. The results of these latter experiments would also suggest that the third Kunitz-type domain and/or the COOH terminus of TFPI may play a role in the interaction of TFPI/Xa binding to the cell surface.

DISCUSSION

Previous studies have definitively shown that the first and second Kunitz-type domains in human TFPI are involved in the neutralization of factor VIIa and factor Xa activity, respectively. The role of the third Kunitz-type domain in TFPI, if any, in these reactions has not been addressed. In the present study, we have compared the abilities of full-length recombinant TFPI, and a COOH-terminal truncated derivative of TFPI lacking the third Kunitz-type domain and COOH-terminal tail, to inhibit cell surface factor VIIa-tissue factor proteolytic activity toward factor X. The results of studies presented here demonstrate that both preparations of TFPI, in the absence and presence of factor X or factor Xa, were kinetically indistinguishable with respect to inhibiting the proteolytic and amidolytic activities of factor VIIa in complex with tissue factor. Furthermore, we provide evidence that the active site serine residues in factor VIIa and factor

Xa are not essential for the interaction of TFPI with these proteases.

Our data also indicate that plasma concentrations of TFPI, in the presence of factor X, was a more effective inhibitor of cell surface factor VIIa-tissue factor than plasma levels of antithrombin III in the absence and presence of 1 unit/ml heparin. Whether its association with plasma lipoproteins reduces the effectiveness of TFPI relative to antithrombin III *in vivo* will require additional studies. In an unexpected finding, antithrombin III was a very effective inhibitor of factor VIIa-tissue factor in the copresence of factor X and heparin, approaching a level of inhibition seen with TFPI and factor X. The mechanism whereby antithrombin III activity is potentiated on the cell surface by the copresence of heparin and factor X is unknown. Active site-mutated factor X (S376A factor X) was as effective as plasma-derived factor X in this reaction, indicating that the active site serine residue of factor Xa was not involved in this mechanism. Furthermore, the use of S376A factor X, as well as the pretreatment of the antithrombin III preparation with anti-TFPI IgG, revealed that the antithrombin III potentiation by heparin and factor X was not due to trace levels of TFPI contaminating either the antithrombin III or plasma-derived factor X preparations used in this study. The presence of heparin in this system was essential, as deletion of heparin resulted in a factor VIIa-tissue factor neutralization rate essentially identical to that observed for antithrombin III alone. Inasmuch as human factor X binds to heparin (34), it is not inconceivable that antithrombin III and factor X (or Xa) occupy distinct sites on heparin, resulting in a ternary complex of antithrombin III-heparin-factor X (Xa) that represents the active anticoagulant species. Factor X (or Xa) in this ternary complex conceivably anchors this complex to a phosphatidylserine-rich site on the J82 cell surface in close proximity to the factor VIIa-tissue factor complex and facilitates rapid neutralization of factor VIIa. Consistent with this hypothesis, preliminary experiments demonstrate that Gla-domainless human factor X does not augment the inhibition of factor VIIa-tissue factor by antithrombin III/heparin.[4]

Both TFPI and $TFPI^{1-161}$ inhibited the amidolytic activity of a factor VIIa-tissue factor complex toward S-2288 at essentially the same rate, confirming our earlier finding (14) using a recombinant TFPI preparation that in all likelihood contained COOH-terminal degraded forms of TFPI (8, 9). In our present system, 10 nM TFPI or 10 nM $TFPI^{1-161}$ inhibited an equimolar concentration of factor VIIa-relipidated tissue factor apoprotein 50% in ~15 min. The rate of inhibition of the factor VIIa-tissue factor amidolytic activity by TFPI in solution phase was, however, markedly faster than the rate of inhibition of factor VIIa-tissue factor proteolytic activity on the cell surface, where 2.5 nM TFPI inhibited factor VIIa-tissue factor activity ~20% in a 60-min incubation period. It is perhaps noteworthy to mention that this rate of cell surface factor VIIa inhibition occurred under conditions where TFPI concentrations (*i.e.* 2.5 nM) were in vast excess of cell surface factor VIIa-tissue factor complexes. The reason(s) for this discordant result is unknown, although it is probable that the J82 cell surface provides unproductive binding sites for TFPI distant from factor VIIa-tissue factor, thereby reducing its effective concentration. Alternatively, in contrast to the amidolytic activity assay conditions, it is possible that the washing steps following the incubation of TFPI with cell surface factor VIIa and preceding the assessment of factor X activation mechanically dissociated some of the factor VIIa-tissue factor-TFPI ternary complex and regenerated factor VIIa-tissue factor reactive sites.

Although initial studies demonstrated that TFPI and $TFPI^{1-161}$ appeared to be indistinguishable in their ability to inhibit preformed complexes of factor VIIa and tissue factor on the J82 cell surface, subsequent studies provided evidence that TFPI and $TFPI^{1-161}$ reproducibly differ in their interaction with the cell surface when in complex with factor Xa. When J82 cell monolayers were treated with TFPI/Xa or $TFPI^{1-161}$/Xa, and subsequently washed and treated with factor VIIa, the extent to which offered factor VIIa could generate factor Xa on the cell surface was markedly higher in cells pretreated with $TFPI^{1-161}$/Xa complexes. This finding suggested that the third Kunitz-type domain and/or COOH-terminal tail of TFPI may contribute to the energy of TFPI/Xa binding to the cell surface. In this regard, Gemmell *et al.* (35), using a glass capillary tube coated with a phospholipid bilayer containing tissue factor apoprotein, reported that tissue factor apoprotein has an independent binding site for factor Xa which facilitated the neutralization of factor VIIa-tissue factor by TFPI. Our data indicate that a preformed complex of TFPI and factor Xa can bind to the J82 cell surface in a reaction that absolutely requires the γ-carboxyglutamic acid domain of factor Xa. This cell-bound TFPI/factor Xa complex is then capable of inhibiting either binding of factor VIIa to tissue factor and/or the expression of factor VIIa-tissue factor activity toward factor X. Our data cannot rule out the possibility of a TFPI·Xa·tissue factor ternary complex on the cell surface, with $TFPI^{1-161}$/Xa exhibiting a reduced affinity for tissue factor relative to TFPI/Xa. However, the results of Callander *et al.* (13), using an ovarian carcinoma cell line (OC-2008) similar to the J82 cell line in its ability to support tissue factor-mediated reactions, suggest that the interaction of TFPI with this cell, in the presence and absence of factor Xa, is considerably more complex than that observed in a well defined capillary tube system. Although the functional consequences of pretreating the OC-2008 cell monolayer with TFPI/Xa was not examined in that study, Callander *et al.* (13) demonstrated distinct factor VIIa-tissue factor-dependent and -independent binding sites for TFPI/Xa complexes on this cell surface. In addition, studies indicated that TFPI/Xa binding to this cell line in the presence of factors VIIa and Xa was not appreciably affected by annexin V, a protein that binds tightly to anionic phospholipids, suggesting that anionic phospholipid was not involved in TFPI/Xa binding to both the tissue factor-dependent and -independent binding sites on OC-2008 cells (13). Accordingly, in the absence of definitive TFPI and $TFPI^{1-161}$ binding studies to the J82 cell monolayers in the presence and absence of factor Xa and factor VIIa, it is difficult to provide a plausible mechanism to explain why TFPI/Xa complexes more effectively prevented the activation of factor X on J82 cell surfaces in comparison with $TFPI^{1-161}$/Xa complexes. Other factors such as increased affinity for the tissue factor-independent sites by $TFPI^{1-161}$/Xa or a more pronounced dissociation of $TFPI^{1-161}$/Xa from the cell surface by the washing steps could also account for its inhibitory effectiveness relative to TFPI/Xa.

It is unclear why $TFPI^{1-161}$ exhibits a 50-fold lower anticoagulant activity than TFPI in a dilute thromboplastin prothrombin time assay,[2] while inhibiting cell surface factor VIIa-tissue factor as effectively as full-length TFPI. As the clotting time in this assay is predicated on the effective concentrations of factor VIIa, factor Xa, and thrombin, we examined the effects of $TFPI^{1-161}$ and TFPI on the amidolytic activities of each of these proteases at equimolar enzyme:inhibitor concen-

[4] T. Hamamoto and W. Kisiel, manuscript in preparation.

trations. As shown in Fig. 3, both TFPI preparations inhibited the amidolytic activity of factor VIIa in complex with soluble tissue factor at essentially the same rate. In agreement with the findings of Broze *et al.* (10), neither TFPI nor $TFPI^{1-161}$ inhibited thrombin amidolytic activity in 2 h of incubation at room temperature. Incubation of either TFPI or $TFPI^{1-161}$ with factor Xa resulted in 50% inhibition of factor Xa amidolytic activity within 1 min by each inhibitor preparation, with complete inhibition occurring in each case in ~2 min of incubation (data not shown). Our data thus indicate that the reactivity of TFPI and $TFPI^{1-161}$ toward factor VIIa and factor Xa are not measurably different and suggest that other factors are at play in determining the potency of full-length TFPI in the clotting assay. Whether TFPI interferes with the interaction of factor VIIa with tissue factor, as suggested by the data in Fig. 6, is unknown and merits further investigation.

Relatively high concentrations of factor VIIa were not very effective in displacing a quaternary complex of factor VIIa-tissue factor-TFPI-factor Xa from the cell surface, with regeneration of only 2% of potential factor VIIa-tissue factor sites. This observation not only underscores the high affinity of the quaternary complex from the cell surface but also raises questions regarding the mechanism of action of plasma-derived and recombinant factor VIIa in restoring hemostasis in hemophilia A patients with circulating antibodies (36–38). Although the J82 cells have provided insight and useful information concerning the mechanisms of physiological clotting, it is difficult at best to directly compare our static J82 cell monolayer system to a dynamic vascular trauma site, particularly when a patient presents for treatment 1–2 h after bleeding commences. Despite this caveat, our displacement studies suggest the possibility that either infused factor VIIa activates factor X in these patients by a tissue factor-independent mechanism or that circulating TFPI-lipoprotein complexes are not as effective in inhibiting cell surface factor VIIa-tissue factor as free uncomplexed TFPI. On the other hand, a small regeneration of factor VIIa-tissue factor sites (1–2%) by infused factor VIIa may be sufficient to eventually arrest bleeding and form a stable hemostatic plug in these patients.

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Kinetics of Factor Xa Inhibition by Tissue Factor Pathway Inhibitor*

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Tissue factor pathway inhibitor is a multivalent, Kunitz-type proteinase inhibitor. It directly inhibits factor Xa and, in a factor Xa-dependent fashion, produces feedback inhibition of the factor VIIa/tissue factor catalytic complex which is responsible for the initiation of coagulation. Human recombinant TFPI (rTFPI) produced in *Escherichia coli* was used to define the kinetic constants describing the human factor Xa:TFPI interaction. The inactivation of factor Xa by *E. coli*-rTFPI is indistinguishable from that of rTFPI produced in mammalian SK-hepatoma cells, suggesting that post-translational modifications such as glycosylation and phosphorylation do not play a major role in the inhibitory process. The slow, tight-binding inhibition of factor Xa follows the scheme:

Slow

$$E + I \underset{k_2}{\overset{k_1}{\rightleftarrows}} EI \overset{k_3}{\underset{k_4}{\rightleftarrows}} EI^*$$

KINETIC MECHANISM

Where the enzyme (E) and inhibitor (I) form an initial, immediate collision complex (EI) that then isomerizes slowly to a tightened final EI^* complex. In the absence of other additions, the initial K_i ($=k_2/k_1$) and final K_i^* for the inhibition of factor Xa by *E. coli*-rTFPI are 1.24 nM and 26.4 pM, respectively. In the presence of calcium ions (5 mM) the interaction between factor Xa and rTFPI is substantially weaker, with a K_i of 42.7 nM and K_i^* of 85.2 pM. The addition of other components of the prothrombinase complex produces enhanced factor Xa inhibition predominantly through an effect on the initial K_i. In the presence of calcium ions and saturating concentrations of phospholipids and factor Va, the K_i and K_i^* for factor Xa inactivation are 2.04 nM and 52.3 pM. The enhancing effect of heparin on the inhibitory process is concentration dependent and exhibits an optimum, reminiscent of the "template" model for heparin's acceleration of thrombin and factor IXa inhibition by antithrombin III. At optimal concentrations, the major mechanism of heparin action is also a reduction in the K_i of the initial encounter complex between factor Xa and rTFPI.

Coagulation is initiated when plasma factor VII or activated factor VII (VIIa) binds to tissue factor, an integral membrane protein expressed constitutively by cells beneath the endothelium of vessels. The factor VIIa-tissue factor complex proteolytically activates factors IX and X resulting ultimately in the formation of a fibrin clot (1–3). Tissue factor pathway inhibitor (TFPI)[1] produces factor Xa-dependent feedback inhibition of the factor VIIa-tissue factor catalytic complex through the formation of a final, quaternary inhibitory complex consisting of factor Xa-TFPI-factor VIIa/tissue factor (4, 5). TFPI is a multivalent protease inhibitor containing three Kunitz-type inhibitory domains (6). Its second Kunitz-type domain is required for binding and inhibiting factor Xa and its first Kunitz-type domain appears to bind and inhibit factor VIIa in the factor VIIa-tissue factor complex (4, 7). The role of the third Kunitz-type domain in TFPI is not known.

TFPI is a potent inhibitor of factor Xa (4) and the prolongation of one-stage plasma coagulation assays initiated by tissue factor (prothrombin time), factor Xa, or the factor X coagulant protein of Russells' Viper Venom (Stypven time) produced by the exogenous addition of full-length TFPI may be predominantly related to its inhibition of factor Xa (8). More than simply the second Kunitz-type domain within the TFPI molecule, however, is required for optimal inhibition of factor Xa by TFPI. Factor Xa inhibition is abrogated following the limited proteolytic cleavage of TFPI produced by human leukocyte elastase that separates the amino terminus and first Kunitz-type domain from the second Kunitz-type domain and the remainder of the molecule (9). Similarly, carboxyl-terminal truncation of the TFPI molecule markedly reduces its factor Xa inhibitory activity (8). Such carboxyl-terminal truncation has been demonstrated in recombinant TFPI produced by cells in tissue culture (8, 10).

Our laboratory has previously reported preliminary studies concerning the kinetics of factor Xa inhibition by the TFPI isolated from the conditioned media of the human hepatoma cell line HepG2 (5). At the time, the potential for carboxyl-terminal truncation of the TFPI molecule was not appreciated, and subsequent studies have shown that the HepG2-TFPI used in these experiments was proteolytically truncated, lacking the positively charged carboxyl terminus of full-length TFPI (residues ~254–276). Here, we report the kinetics of factor Xa inhibition by full-length rTFPI in the presence and absence of components of the prothrombinase complex and heparin.

EXPERIMENTAL PROCEDURES

Materials—*p*-Nitrophenyl-*p*-guanidinobenzoate hydrochloride and rabbit brain cephalin (phospholipids) were obtained from Sigma. The chromogenic substrate S2765 (*N*-α-Cbo-D-Arg-Gly-Arg-*p*NA) for factor Xa was purchased from Chromogenic AB (Molndal, Sweden) and Chromozyme TH (tosyl-Gly-Pro-Arg-A-NA) for thrombin from Boehringer Mannheim. Both substrates were dissolved in water and stored at 4 °C in the dark before dilution into 150 mM NaCl, 50 mM Tris-HCl, pH 7.5,

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[1] The abbreviations used are: TFPI, tissue factor pathway inhibitor; rTFPI, recombinant TFPI; PAGE, polyacrylamide gel electrophoresis.

with 0.1% bovine serum albumin (TBSA) directly prior to use. Heparin sodium was from Lyphomed, Division of Fujisawa USA, Inc.

Proteins—Recombinant TFPI (rTFPI) produced in *E. coli*[2] and the human hepatoma cell line SK-Hep were isolated as described (11). The mass of *E. coli*-rTFPI was determined by amino acid analysis and the mass of SK-rTFPI was determined by assuming 1:1 stoichiometry of the SK-rTFPI:factor Xa interaction (8). Human factor X was isolated and activated as previously described (4), and the factor Xa quantitated by active-site titration with *p*-nitrophenyl-*p*-guanidinobenzoate hydrochloride (12). Human factor Va was purchased from Haematologic Technologies (Essex Junction, VT). Prothrombin was isolated as previously described (4).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. SDS-PAGE was performed using a 15% separating gel and 4% stacking gel (8). Western blotting was performed as previously described using specific antipeptide antibodies directed against the NH_2 terminus (residues 1–12) and COOH terminus (residues 265–276) of the TFPI molecule (8).

Kinetic Methods—In independent experiments (not shown), saturating concentrations of phospholipids and factor Va for factor Xa inhibition by rTFPI were determined to be a 1:10 dilution of the stock rabbit brain cephalin (prepared as suggested by the manufacturer) and 18.7 nM, respectively. Calcium chloride was used at 5 mM final concentration and reactions were performed at room temperature (21 °C).

The Michaelis-Menten constants (K_m) for the hydrolysis of S2765 by factor Xa were determined using standard methods: absence of other additions, 52.7 ± 1.6 μM; with calcium ions, 51.5 ± 1.6 μM; with calcium and phospholipids, 42.6 ± 2.3 μM; with calcium, phospholipids, and factor Va, 82.9 ± 0.4 μM; with calcium and 10 units/ml heparin, 33.7 ± 2.3 μM; with calcium, phospholipids, and 1 unit/ml heparin, 36.9 ± 0.5 μM; and with calcium, phospholipids, factor Va, and 1 unit/ml heparin, 68.1 ± 1.2 μM.

Kinetic constants which describe the inhibition of factor Xa by TFPI were determined as previously described (13–18). Reactions (1.0 ml final volume) were initiated by the addition of factor Xa (0.2 nM) to mixtures of rTFPI (2–40 nM), additional components, and the chromogenic substrate S2765 (typically 250 or 500 μM) in TBSA. Progress curves reflecting the increase in absorbance at 405 nm over time were monitored until a steady-state velocity was achieved (~30 min). Data for each progress curve (10 points/minute) were collected on a Response spectrophotometer (Gilford, Corning Laboratory Sciences Co., Oberlin, OH) and kinetic parameters (see below) were derived using a nonlinear regression curve fit program (Sigma Plot, Jandel Scientific, Corte Modera, CA). The coefficient of variation for each of the fitted parameters in the experiments presented was consistently <5%.

The effect of rTFPI on the cleavage of prothrombin by the prothrombinase complex was examined using prothrombin (1.4 μM), factor Xa (0.2 nM), $CaCl_2$ (5 mM), saturating concentrations of factor Va and phospholipids (as above), and various concentrations of *E. coli*-rTFPI (0, 2, and 20 nM). Reactions were initiated by the addition of factor Xa to mixtures containing the other components and frequent 20-μl samples were removed, diluted with 900 μl of TBSA containing 5 mM EDTA, and assayed for thrombin using the chromogenic substrate Chromozyme TH (final concentration 100 μM). The factor Xa carried over to the thrombin assays produced negligible cleavage of Chromozyme TH.

RESULTS

Proteolytic, carboxyl-terminal truncation of TFPI occurs during its production in tissue culture cells (8, 10). Although the enzyme(s) responsible for this proteolysis has not been identified, the cleavage occurs within a stretch of basic amino acid residues (TFPI residues 254–261) and produces a truncated form of TFPI that lacks the carboxyl-terminal 15–20 amino acids of full-length TFPI and that possesses considerably less factor Xa inhibitory activity (8). Our laboratory has previously reported a preliminary analysis of the kinetics of factor Xa inhibition by TFPI produced in the human hepatoma cell line HepG2 (5). Subsequent studies, however, revealed that the HepG2-TFPI used for those studies was truncated at the carboxyl terminus. Thus, sufficient full-length rTFPI was produced in a prokaryotic expression system to repeat the kinetic analysis of factor Xa inhibition. In initial experiments, the kinetic results with *E. coli*-rTFPI were compared to those of rTFPI produced by SK-hepatoma cells, which is available in only limited quantities. Based on SDS-PAGE (Fig. 1) and Western blotting using antibodies that recognize the NH_2 terminus and COOH terminus of TFPI (not shown) the *E. coli*- and SK-rTFPI used for these studies were estimated to be greater than 90% homogeneous and to consist of greater than 90% full-length TFPI molecules. *E. coli*-rTFPI has an apparent molecular weight of 32,000, whereas the molecular weight of SK-rTFPI is 42,000, presumably reflecting the absence of carbohydrate in *E. coli*-rTFPI. Similar to the TFPI produced from other sources (4, 8), *E. coli*-rTFPI bound active site-titrated factor Xa with 1:1 stoichiometry (data not shown).

Mechanism of Enzyme Inhibition—The addition of factor Xa to substrate (S2765) in the presence of rTFPI results in slow inhibition of enzymatic activity with a gradual progression toward a steady-state reaction velocity. At given concentrations of reactants, the final steady-state velocity was the same whether the enzyme was added last or the enzyme and rTFPI were preincubated and the substrate added last (Fig. 2). This type of slow, reversible inhibition can be explained by one of the following reaction schemes:

Slow

$$E \underset{k_2}{\overset{k_1}{\rightleftarrows}} EI \qquad \text{MECHANISM I}$$

Slow

$$E \underset{k_2}{\overset{k_1}{\rightleftarrows}} EI \underset{k_4}{\overset{k_3}{\rightleftarrows}} EI^* \qquad \text{MECHANISM II}$$

Slow

$$E \underset{k_2}{\overset{k_1}{\rightleftarrows}} E^* \underset{k_4}{\overset{k_3}{\rightleftarrows}} E^*I \qquad \text{MECHANISM III}$$

In Mechanism I, the inhibitor reacts slowly with the enzyme, while Mechanism II describes a mechanism in which the enzyme and inhibitor react immediately to form an initial collision complex followed by a slow isomerization to a tightened EI^* complex. Mechanism III involves a slow isomerization of the enzyme itself, which then interacts with the inhibitor. The differentiation of these three mechanisms is complicated by the fact that the enzyme-inhibitor complex approaches steady-state at a much slower rate than the enzyme-substrate interaction. Therefore, steady-state rate equations designed for the determination of kinetic constants for conventional competitive inhibitors are inadequate and specific mathematical formulations are used for appropriate analysis (13–18).

Data from experiments performed under presumed pseudo-

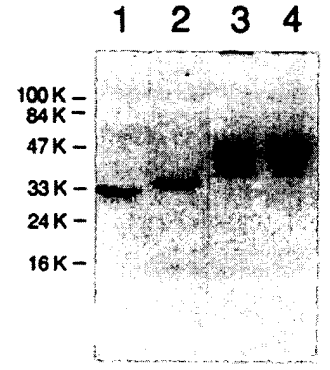

FIG. 1. **SDS-PAGE of rTFPI.** *Lane 1*, unreduced *E. coli*-rTFPI (2 μg); *lane 2*, 2-mercaptoethanol reduced *E. coli*-rTFPI (2 μg); *lane 3*, unreduced SK-rTFPI (10 μg); *lane 4*, 2-mercaptoethanol reduced SK-rTFPI (10 μg). Proteins were stained with Coomassie Brilliant Blue.

[2] T.-C. Wun, manuscript in preparation.

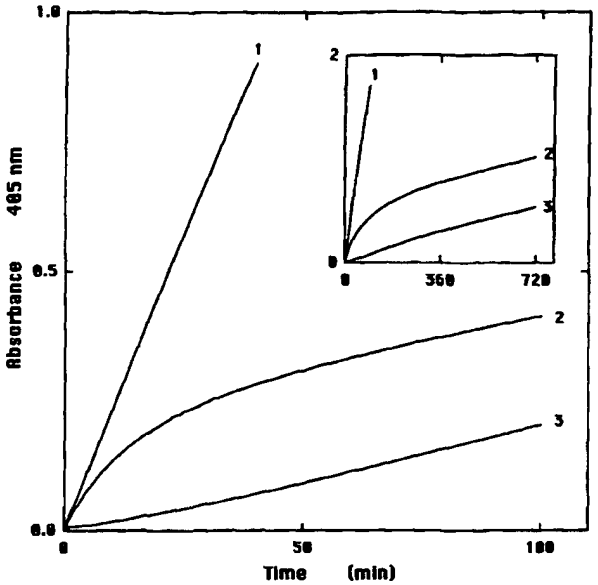

FIG. 2. **Slow tight-binding, reversible inhibition of factor Xa by rTFPI.** Three sets of reactions were constructed: 1) factor Xa was added to S2765 alone; 2) factor Xa was added to a mixture of rTFPI and S2765; and 3) factor Xa and rTFPI were incubated for 1 h, then S2765 was added to the mixture. Concentrations of factor Xa, rTFPI, and S2765 were 0.2 nM, 2 nM, and 0.25 μM, respectively. Reactions were monitored at 405 nm for ≥100 min at room temperature. *Main figure*, *E. coli*-rTFPI; *inset*, SK-rTFPI.

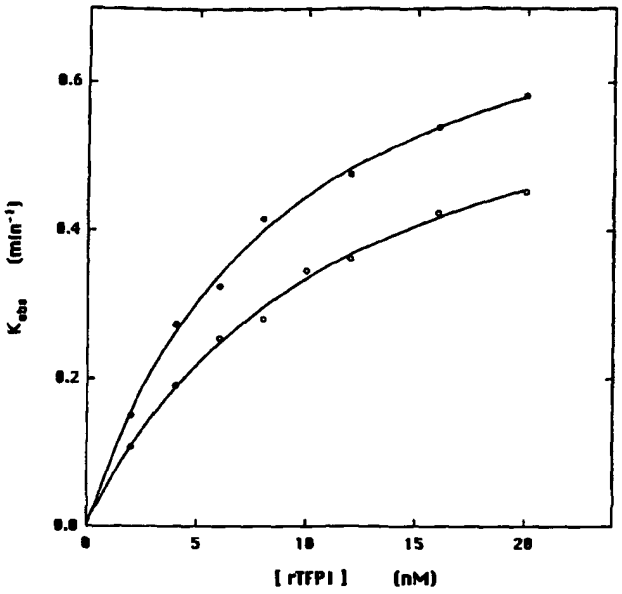

FIG. 3. **Dependence of k_{obs} on rTFPI concentration.** Values for k_{obs} (S2765 = 500 μM) were generated from data fitted to Equation 1 and the plotted points were fitted to Mechanism II (see text). *Open circles*, *E. coli*-rTFPI; *closed circles*, SK-rTFPI.

first order conditions and initiated by the addition of enzyme to substrate and inhibitor were fitted by nonlinear regression analysis to the integrated rate equation for slow binding inhibition (18):

$$A = v_s t + (v_o - v_s)\{1 - \exp(-k_{obs} t)\}/k_{obs} + A_o \qquad \text{(Eq. 1)}$$

where A is absorbance at 405 nm at time t; v_s, final steady-state velocity; v_o, initial velocity; k_{obs}, apparent rate constant for the transition from v_o to v_s; A_o, initial absorbance at 405 nm. Fitting yields values for v_o, v_s, k_{obs}, and A_o for each progress curve. These values are then transformed in various ways to generate the rate and inhibitory constants for the reaction.

The value of the apparent rate constant (k_{obs}) for each of the potential mechanisms for slow, reversible inhibition listed above is related to the individual kinetic constants as follows (17):

$$k_{obs} = k_2 + k_1 [I]/(1 + [S]/K_m) \qquad \text{MECHANISM I}$$

$$k_{obs} = k_4 + k_3 [I]/([I] + K_i (1 + [S]/K_m)) \qquad \text{MECHANISM II}$$

$$k_{obs} = k_3/(1 + [S]/K_m) + (k_4)(K_i)/([I] + K_i) \qquad \text{MECHANISM III}$$

where [S] is the concentration of chromogenic substrate and K_m is the Michaelis-Menten constant describing the interaction of the enzyme with the substrate.

Experiments showed that the apparent rate constant (k_{obs}) for the factor Xa:rTFPI interaction is a hyperbolic function of [rTFPI] (Fig. 3), and that v_o is inversely related to [rTFPI] (not shown). Thus, the kinetic mechanism described by Mechanism I can be excluded as it predicts a linear relationship between k_{obs} and [rTFPI] and that v_o is independent of [rTFPI] (18, 19). Mechanism III can also be discounted because k_{obs} increases with increasing [rTFPI], which would not be the case with this mechanism (18–20). We conclude therefore that inhibition of factor Xa by rTFPI is best described by Mechanism II, which is typical of Kunitz-type proteinase inhibitors (21).

Determination of Kinetic Constants—To determine the kinetic constants for the inhibition of factor Xa by *E. coli*- and

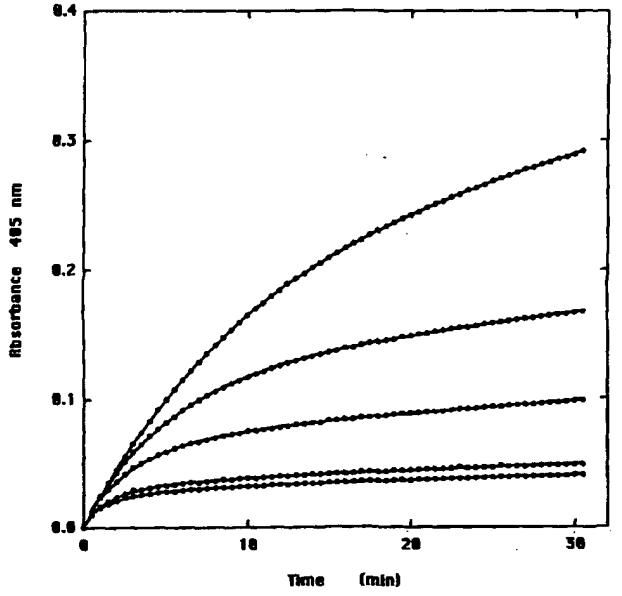

FIG. 4. **Progress curves for factor Xa inhibition by various concentrations of *E. coli*-rTFPI.** Factor Xa (0.2 nM) was added to reactions containing S2765 (500 μM) and rTFPI. *Curves* from top to bottom are from reactions with 2, 4, 8, 16, and 20 nM *E. coli*-rTFPI. *Closed circles* represent the experimental data (only every 30-s values are plotted); *lines* represent curves derived from fitting the experimental data to Equation 1 (see text).

SK-rTFPI, a series of progress curves were developed using 0.2 nM factor Xa, 500 μM S2765, and varying concentrations of rTFPI (2–20 nM) (Fig. 4). The correlation between the experimental data and the fitted curves is excellent.

In Mechanism II, the enzyme and inhibitor form an immediate encounter complex with the inhibitory constant K_i ($=k_2/k_1$), which "slowly" isomerizes in what may be a multistep process (21) to a "tightened" EI^* complex with a final $K_i^* = (K_i)(k_4)/(k_3 + k_4)$ (19). The rate constant k_4, describing the return

from the tightened complex EI^* to EI, was calculated to be 0.0161 ± 0.0017 and 0.0181 ± 0.0037 for *E. coli*-rTFPI and SK-rTFPI, respectively, using the relationship (22):

$$k_4 = (k_{obs})(v_s)/v_o \qquad \text{(Eq. 2)}$$

A subsequent plot of $1/(k_{obs} - k_4)$ *versus* 1/[TFPI] (Fig. 5) yields values for k_3 (y intercept = $1/k_3$) and K_i (x intercept = $-(1 + [S]/K_m)/K_i$). The derived kinetic constants for the inhibition of factor Xa by *E. coli*-rTFPI and SK-rTFPI are listed in Table I. Plots of V_{max}/v_o and V_{max}/v_s *versus* [I] provide an alternative means for the calculation of K_i and K_i^*, respectively (16, 18). The kinetic constants calculated with this method are in close agreement with those shown in Table I; K_i and K_i^* of 1.12 nM and 29.95 pM for *E. coli*-rTFPI and 0.82 nM and 24.45 pM for SK-rTFPI. These forms of rTFPI appear to possess nearly equivalent inhibitory activity and are potent inhibitors of factor Xa in the absence of other cofactors.

Effects of Components of the Prothrombinase Complex on Factor Xa Inhibition by TFPI—Factor Xa is the enzymatic component of the prothrombinase complex that also contains calcium ions, phospholipids, and activated factor V (factor Va). Thus, the effect of the various components of the prothrombinase complex on factor Xa inhibition by TFPI is of interest. The kinetic constants derived for the inhibition of factor Xa by rTFPI in the presence of calcium ions (5 mM), calcium ions and phospholipids (1:10 dilution of stock rabbit brain cephalin), and calcium ions, phospholipids, and factor Va (18.7 nM) are shown in Table II. The presence of calcium ions reduces the affinity of the initial encounter complex (K_i) over 30-fold and that of the final tightened complex 3-fold. Preliminary data (23) suggest that this effect of calcium ions may be due to the interruption of a direct interaction between the positively charged carboxyl terminus of TFPI and the negatively charged γ-carboxyglutamic acid domain of factor Xa (see "Discussion").

With the sequential addition of the other components of the prothrombinase complex along with the calcium ions, there is a progressive decrease in the K_i which is 43 nM with calcium ions alone, 15 nM with calcium ions and phospholipids, and 2 nM with calcium ions, phospholipids, and factor Va (Table II). Thus, the predominant effect of these cofactors is to increase the affinity of the initial, collision complex between factor Xa and TFPI.

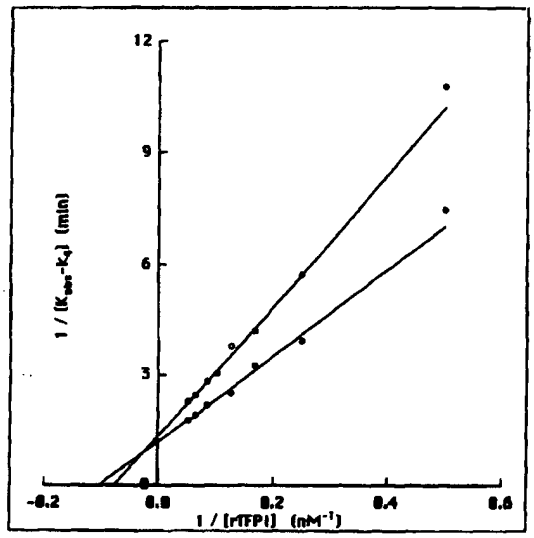

FIG. 5. **Plots of $1/(k_{obs}-k_4)$ *versus* 1/[rTFPI] to obtain values for K_i and k_3.** Data from the progress curves (S2765 = 500 μM) used to generate k_{obs} in Fig. 4 were also used to derive values for k_4 (Equation 2, see text). The y intercept in the graph represents $1/k_3$ and the x intercept is $-(1 + [S]/K_m)/K_i$. *Open circles, E. coli*-rTFPI; *closed circles*, SK-rTFPI.

TABLE I
Kinetic constants for rTFPI inhibition of Factor Xa

	K_i	K_i^*	k_3	k_4
	nM	pM	min^{-1}	
E. coli-rTFPI	1.24	26.4	0.741	0.0161
SK-rTFPI	0.93	19.8	0.833	0.0181

TABLE II
Kinetic constants for E. coli-rTFPI inhibition of factor Xa with calcium ions (Ca^{2+}), phospholipids (PI), factor Va (Va), and heparin (Hep)

	Hep	K_i	K_i^*	k_3	k_4
	units/ml	nM	pM	min^{-1}	
Ca^{2+}	0	42.7	85.2	2.50	0.0050
Ca^{2+}/PI	0	14.6	65.2	1.18	0.0053
Ca^{2+}/PI/Va	0	2.04	52.3	0.324	0.0086
Ca^{2+}	10	3.13	30.9	3.077	0.0307
Ca^{2+}/PI	1	1.28	17.9	1.39	0.0197
Ca^{2+}/PI/Va	1	0.79	21.8	0.488	0.0138

The ability of TFPI to inhibit the prothrombinase complex in the presence of prothrombin was also tested. Factor Xa (0.2 nM final concentration) was added to reactions containing prothrombin (1.4 μM = plasma concentration), factor Va (18.7 nM), phospholipids (1:10 stock rabbit brain cephalin), $CaCl_2$ (5 mM), without or with *E. coli*-rTFPI (2 nM or 20 nM). The initial rate of thrombin generation was reduced 36% by 2 nM rTFPI and 85% by 20 nM rTFPI (data not shown). These results are in good agreement with those predicted by assuming an initial K_i of *E. coli*-rTFPI for the prothrombinase complex of 2 nM (Table II) and a K_m of prothrombin for the prothrombinase complex of 1 μM (24).

The Effect of Heparin—In the presence of calcium ions, heparin appears to enhance the inhibition of factor Xa by TFPI (23). The response to heparin is concentration-dependent and exhibits an optimum, reminiscent of the putative "template" effect of heparin on the interaction between thrombin and antithrombin III (25). Attempts to determine the kinetic effects of heparin on the factor Xa:TFPI interaction, however, were not straightforward. Preliminary studies showed that the concentration of heparin required for the optimal enhancement of factor Xa inhibition by TFPI in the presence of calcium ions is dependent on the concentration of the substrate S2765; at 250 μM S2675, the optimum heparin concentration is 10 units/ml, whereas with 50 μM S2765 it is 0.1–1 unit/ml (Fig. 6, and data not shown). A similar although less pronounced effect was found when the alternative substrate Spectrozyme Xa (MeO-CO-D-CHG-Gly-Arg-pNA, American Diagnostica, Greenwich, CT) was substituted for S2765 (not shown). The phenomenom appears most likely related to the binding of heparin by the substrates and is more marked with S2765 (*N*-α-Cbo-D-Arg-Gly-Arg-*p*NA) which contains 2 basic amino acids. The optimum concentration of heparin (at 250 μM S2765) was also dependent on the cofactors present within the reactions. With calcium ions alone, calcium ions/phospholipids, and calcium ions/phospholipids/factor Va, the enhancement of factor Xa inhibition by TFPI was greatest with 10, 1–10, and 1 unit/ml heparin, respectively (Fig. 6).

The derived kinetic constants for TFPI inhibition of factor Xa in the presence of various components of the prothrombinase complex and optimal concentrations of heparin are shown in Table II. It appears that the predominant effect of heparin is to increase the affinity of the initial complex between factor Xa and TFPI (K_i). Notably, the presence of heparin also increased the dissociation rate constant (k_4) of the final tightened EI^* complex.

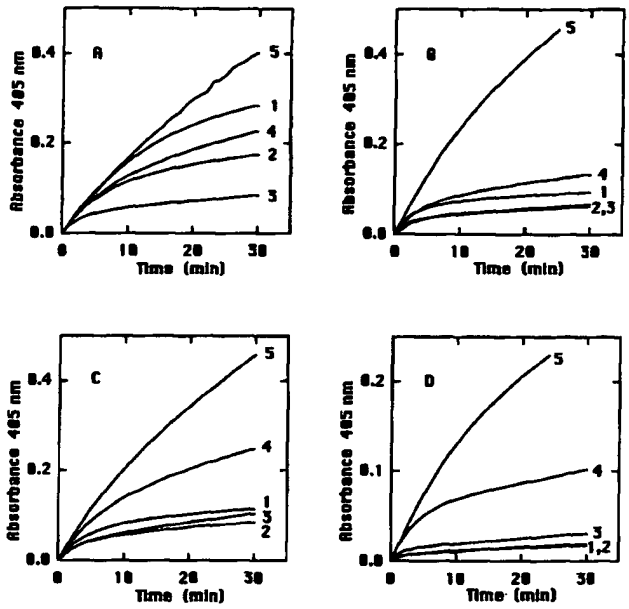

FIG. 6. **Optimal heparin concentration for factor Xa inhibition by rTFPI depends on the concentration of S2765 and the presence of cofactors.** All reactions contained 0.2 nM factor Xa, 4.0 nM *E. coli*-rTFPI, and 5 mM $CaCl_2$. Additional reaction components: *panel A*, S2765 (250 μM); *panel B*, S2765 (250 μM) and 1:10 stock rabbit brain cephalin; *panel C*, S2765 (250 μM), 1:10 stock rabbit brain cephalin and factor Va (18.7 nM); *panel D*, S2765 (50 μM). In each panel, *curves 1, 2, 3, 4,* and *5* stand for 0.1, 1, 10, 100, and 1000 units/ml heparin, respectively.

DISCUSSION

In addition to inhibiting the factor VIIa-tissue factor catalytic complex in the presence of factor Xa, TFPI is a potent inhibitor of factor Xa itself (4). Here, we have determined the kinetic mechanism and constants for factor Xa inhibition by TFPI in the absence and presence of the other components of the prothrombinase complex. These *in vitro* studies were performed at room temperature (21 °C), and the kinetics of factor Xa inhibition by TFPI may be enhanced *in vivo* (37 °C).

Recombinant TFPI produced in *E. coli* has been used for these experiments, as it is available in substantial quantities. Moreover, the activity of the *E. coli* derived material appears comparable to that of rTFPI produced in SK hepatoma mammalian cells suggesting that post-translational modifications of the TFPI molecule, such as glycosylation and phosphorylation, do not play a major role in its interaction with factor Xa (26, 27).

The addition of calcium ions markedly slows the inhibition of factor Xa by TFPI. This effect is mediated predominantly through a reduction in the affinity of the initial collision complex between factor Xa and TFPI (K_i). These results are consistent with separate studies using altered forms of rTFPI which suggest that, in the absence of other additions, the positively charged carboxyl terminus of TFPI interacts with the negatively charged γ-carboxyglutamate (Gla) domain of factor Xa or a conformation of factor Xa that requires the absence of calcium ions (23). The binding of Ca^{2+} by the Gla domain of factor Xa would prevent its ionic interaction with the basic carboxyl-terminal region of TFPI. Whatever the mechanism for the inhibitory effect of calcium ions on the inactivation of factor Xa by TFPI, it is important to note that calcium ions will be present in areas where factor Xa and TFPI are likely to interact *in vivo*.

The addition of the phospholipids, and phospholipids and factor Va to reactions containing calcium ions produces a progressive enhancement in the rate of factor Xa inhibition by TFPI. The major kinetic mechanism responsible for this effect is a reduction in the K_i of the initial factor Xa-TFPI complex. Although previous qualitative data suggests that the environment about the active site of factor Xa is influenced when factor Xa is bound to components of the prothrombinase complex (28–30), these changes are associated with only a minimal effect on the catalytic activity of the active site against small molecular weight substrates (31). Thus, the mechanism(s) for the increased affinity of the initial factor Xa-TFPI complex noted with the addition of phospholipids or phospholipids and factor Va to calcium ions likely involves changes within the factor Xa molecule distant from its active site, conformational effects upon TFPI, and/or the simultaneous interaction of TFPI with factor Xa and the other prothrombinase components. These results are consistent with the notion that the interaction between factor Xa and TFPI involves considerably more than simply the binding of the active site of factor Xa by the second Kunitz-domain of TFPI (8, 9, 23, 32). The demonstrated high affinity of TFPI for factor Xa in the presence of the other prothrombinase components (K_i = 2 nM) is also consistent with the supposition that much of the apparent anticoagulant activity measured in one-stage coagulation assays following the addition of exogenous full-length TFPI to plasma may be related to the inhibition of factor Xa action (8, 23).

The effect of heparin on the inactivation of factor Xa by TFPI in the presence of calcium ions is concentration-dependent and high concentrations of heparin inhibit the process (Ref. 23 and this report). The pattern of this response is reminiscent of the template mechanism thought to be involved in heparin's acceleration of thrombin and factor IXa inhibition by antithrombin III (25). In a similar fashion, heparin may enhance the affinity of the interaction between factor Xa and TFPI by simultaneously binding both members of the complex. Interestingly, the bridging of antithrombin III and factor Xa by heparin does not appear to play a major role in the heparin-mediated acceleration of factor Xa inhibition by antithrombin III (33–38). Instead, a conformational change in antithrombin III induced by its binding to a specific pentasaccharide in heparin appears most important for factor Xa inhibition (39–41). A low molecular weight heparin of insufficient length to accelerate antithrombin III inactivation of thrombin does enhance factor Xa inhibition by TFPI, although to a lesser degree than standard heparin.[3] Thus the overall effect of heparin on the factor Xa: TFPI interaction may involve a conformational change in TFPI (and/or factor Xa) associated with heparin binding, as well as the provision of a template for the simultaneous binding of both factor Xa and TFPI. Studies designed to further delineate the effects of heparin on factor Xa inhibition by TFPI are in progress.

Normal plasma concentrations of TFPI are low (2 nM) and recent studies have shown that only a fraction of the circulating TFPI represents the full-length molecule.[4] The remainder of the TFPI in plasma is made up of carboxyl-terminal truncated forms that possess much less anti-factor Xa activity (8, 23). Thus, under physiologic conditions, the modulation of prothrombinase activity by TFPI is likely to be modest and of limited extent. The combination of increased concentrations of circulating full-length TFPI (42, 43) and increased inhibitory potency of TFPI that follows heparin infusion, however, suggest that TFPI may be responsible for a substantial portion of the plasma anti-factor Xa activity that accompanies heparin therapy (44). Moreover, when full-length TFPI is used as a therapeutic agent, its concentration in plasma may reach over

[3] G. J. Broze, unpublished observations.

[4] G. J. Broze, G. Lange, and L. MacPhail, manuscript in preparation.

20-fold that in normal plasma. Under these conditions, TFPI-mediated inhibition of prothrombinase could be substantial and may contribute to the antithrombotic effects of TFPI infusion (45, 46).

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Biochemistry

Molecular cloning, expression, and partial characterization of a second human tissue-factor-pathway inhibitor

(blood coagulation/Kunitz-type inhibitors)

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ABSTRACT **Previous studies have shown that tissue-factor-pathway inhibitor (TFPI) is an important regulator of the extrinsic pathway of blood coagulation through its ability to inhibit factor Xa and factor VIIa–tissue factor activity. We describe the molecular cloning and expression of a full-length cDNA that encodes a molecule, designated TFPI-2, that has a similar overall domain organization and considerable primary amino acid sequence homology to TFPI. After a 22-residue signal peptide, the mature protein contains 213 amino acids with 18 cysteines and two canonical N-linked glycosylation sites. The deduced sequence of mature TFPI-2 revealed a short acidic amino-terminal region, three tandem Kunitz-type domains, and a carboxyl-terminal tail highly enriched in basic amino acids. Northern analysis indicates that TFPI-2 is transcribed in umbilical vein endothelial cells, liver, and placenta. TFPI-2 was expressed in baby hamster kidney cells and purified from the serum-free conditioned medium by a combination of heparin-agarose chromatography, Mono Q FPLC, Mono S FPLC, and Superose 12 FPLC. Purified TFPI-2 migrated as a single band in SDS/PAGE and exhibited a molecular mass of 32 kDa in the presence and absence of reducing agent. The amino-terminal sequence of recombinant TFPI-2 was identical to that predicted from the cDNA. Despite its structural similarity to TFPI, the purified recombinant TFPI-2 failed to react with polyclonal anti-TFPI IgG. Preliminary studies indicated that purified recombinant TFPI-2 strongly inhibited the amidolytic activities of trypsin and the factor VIIa–tissue factor complex. In addition, the inhibition of factor VIIa–tissue factor amidolytic activity by recombinant TFPI-2 was markedly enhanced in the presence of heparin. TFPI-2 at high concentrations weakly inhibited the amidolytic activity of human factor Xa, but had no measurable effect on the amidolytic activity of human thrombin.**

Tissue-factor-pathway inhibitor (TFPI) plays a key role in the regulation of tissue factor-initiated blood coagulation (1, 2). Human TFPI is a trace 42-kDa plasma glycoprotein that is synthesized primarily by endothelial cells (3) and consists of a negatively charged amino-terminal region, three tandem Kunitz-type inhibitor domains, and a highly basic carboxyl-terminal tail (4). Site-directed mutagenesis studies revealed that the second Kunitz-type domain of TFPI forms a complex with factor Xa and inhibits its amidolytic and proteolytic activity (5). The factor Xa–TFPI complex rapidly inhibits activity of the factor VIIa–tissue factor complex through the interaction of the first Kunitz-type domain in TFPI and the active site of factor VIIa (5). Although TFPI has been shown to inhibit factor VIIa–tissue factor amidolytic (6, 7) and proteolytic (7, 8) activity independent of factor Xa, it is unclear whether TFPI exerts any meaningful inhibitory activity against factor VIIa–tissue factor *in vivo* in the absence of factor Xa. While the roles of the first and second Kunitz-type domains in the mechanism of action of TFPI are clear from several studies, mutagenesis of the P_1 position of the third Kunitz-type domain in TFPI failed to produce detectable changes in the inhibitory properties of the protein (5). The presence and/or integrity of the highly basic carboxyl-terminal region is essential for full anticoagulant activity in dilute thromboplastin prothrombin time assays (9) and optimal inhibition of factor Xa (10).

In light of the apparent physiological significance of TFPI (11), as well as the documented homology between its three Kunitz-type domains and the superfamily of Kunitz inhibitors (4), we initiated a program to isolate additional members of this inhibitor family from a variety of human tissues. Using an oligonucleotide probe designed around the most highly conserved amino acid sequence in this superfamily, we previously observed that, under low-stringency hybridization conditions, additional hybridizing bands were observed on a Northern blot of mRNA from several tissues including liver, pancreas, and placenta. The first report of these studies described the isolation and characterization of a homolog of the human amyloid precursor protein (12), which was identified from this approach by virtue of its internal Kunitz-type inhibitor domain. We now report the isolation and characterization of the cDNA for another Kunitz-type inhibitor with striking overall domain organization similarity and considerable primary sequence homology to TFPI.§ In view of its similarity to TFPI, we call this protein TFPI-2. We also describe the expression and purification of recombinant TFPI-2 and report that TFPI-2 readily inhibits factor VIIa–tissue factor amidolytic activity in a reaction that is significantly enhanced by low levels of heparin.

MATERIALS AND METHODS

Materials. Mono Q, Mono S, Superose 12, and SDS/PAGE low molecular weight standards were obtained from Pharmacia. Benzoyl-Ile-Glu-Gly-Arg-*p*-nitroanilide (S-2222), D-Phe-Pip-Arg-*p*-nitroanilide (S-2238), D-Ile-Pro-Arg-*p*-nitroanilide (S-2288), and D-Val-Leu-Lys-*p*-nitroanilide (S-2251) were from Helena Laboratories. L-1-Tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin was from Worthington. All other reagents were the best grade commercially available.

Human factor Xa, human thrombin, S376A factor X (recombinant human factor X with the active-site Ser-376 replaced by alanine), and rabbit anti-human TFPI IgG were prepared as described (6, 7, 13). Recombinant human factor

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Abbreviations: TFPI, tissue-factor-pathway inhibitor; S376A factor X, recombinant mutant human factor X with alanine substituted for Ser-376 at the active site.

‡To whom reprint requests should be addressed.

§The sequence reported in this paper has been deposited in the GenBank data base (accession no. L27624).

VIIa was prepared as described (14) and kindly provided by Ulla Hedner (Novo–Nordisk, Copenhagen). Full-length recombinant human tissue factor apoprotein was prepared as described (15) and generously provided by Gordon Vehar (Genentech). Tissue factor apoprotein was relipidated as described (7). All proteins were homogeneous as judged by SDS/PAGE (16).

cDNA Isolation and Sequencing. A human placenta cDNA library (Clontech, no. HL-10756) was screened with an antisense 30-mer oligonucleotide, 5′-GTTGTTGCTGT-TGCCTCCGCAGCCTCCGTA-3′. Plaques (2.4×10^6) were screened at low hybridization stringency. Nylon membranes were hybridized with probe at 55°C in 5× SSPE (1× is 0.15 M NaCl/10 mM NaH_2PO_4/1 mM EDTA, pH 7.4)/5× Denhardt's solution/0.5% SDS containing salmon sperm DNA (100 μg/ml). Filters were washed at 60°C in 2× standard saline citrate with 0.1% SDS. Positive plaques were purified and the largest *Eco*RI insert was subcloned into pUC19 and sequenced (17).

Mammalian Cell Expression. The full-length cDNA for TFPI-2 was directionally cloned into expression vector Zem229R and expressed in BHK cells (18).

Trypsin Inhibitor Assay. Samples (20–100 μl) of conditioned medium from transfected BHK cells were added to 300 μl of trypsin (2.4 μg/ml) in 50 mM Tris·HCl, pH 7.5/100 mM NaCl. After incubation for 30 min at room temperature, 20 μl of 10 mM S-2251 was added and residual trypsin activity was measured at 405 nm.

Purification of Recombinant TFPI-2. Recombinant TFPI-2 was purified from conditioned BHK medium by sequential chromatography on heparin-agarose, Mono Q, Mono S, and Superose 12. Serum-free medium (≈5 liters) was adjusted to pH 7.5, filtered through a 0.22-μm filter, and applied to a column (2.6 × 35 cm) of heparin-agarose equilibrated at 4°C with 50 mM Tris·HCl, pH 7.5/10% (vol/vol) glycerol (buffer A) at 3 ml/min. The column was washed with buffer A/0.2 M NaCl. TFPI-2 activity, as judged by its ability to inhibit trypsin, was eluted from the column with 1 M NaCl in buffer A. The 1 M NaCl eluent was then dialyzed at 4°C against 25 mM Tris·HCl, pH 7.5/10% glycerol and the retentate was applied at room temperature to a Mono Q HR 5/5 column equilibrated with 25 mM Tris·HCl, pH 7.5/10% glycerol. TFPI-2 was eluted from the column in a linear gradient of 0–0.5 M NaCl. Mono Q TFPI-2 fractions were pooled, dialyzed against 25 mM sodium citrate, pH 5.0/10% glycerol, and applied at room temperature to a Mono S HR 5/5 column at 0.5 ml/min. TFPI-2 activity was eluted in a gradient composed of 25 mM sodium citrate, pH 5.0/10% glycerol and 25 mM Tris·HCl, pH 7.5/10% glycerol/1 M NaCl. Fractions from the Mono S column containing TFPI-2 activity were pooled, concentrated to ≈1 ml by ultrafiltration, and subjected to Superose 12 FPLC at room temperature in 50 mM Tris·HCl, pH 7.5/100 mM NaCl. Fractions eluted from the Superose 12 column with TFPI-2 activity were subjected to SDS/PAGE, and pure fractions were pooled and stored at −80°C.

Effect of Recombinant TFPI-2 on the Amidolytic Activities of Factor VIIa–Tissue Factor, Factor Xa, and Thrombin. Relipidated recombinant tissue factor apoprotein (20 nM effective concentration) and recombinant factor VIIa (20 nM) were coincubated in a 96-well microtitration plate in 50 μl of 10 mM Hepes, pH 7.45/137 mM NaCl/4 mM KCl/11 mM glucose/5 mM $CaCl_2$/0.5% bovine serum albumin (buffer B). After a 5-min incubation at 37°C, 50 μl of either TFPI-2 (0–400 nM), TFPI-2 (0–400 nM)/S376A factor X (200 nM), or TFPI-2 (0–400 nM)/heparin (0.4 unit/ml) dissolved in buffer B was added to the well and incubated for 15 min at 37°C. At this point, 50 μl of S-2288 (3 mM) was added to the well and A_{405} was determined in a kinetic microplate reader (Molecular Devices; model UVmax). The inhibition of 10 nM human factor Xa and 10 nM human thrombin by various concentrations of recombinant TFPI-2 was assessed separately in this system using S-2222 and S-2238, respectively, as chromogenic substrates.

RESULTS

Molecular Cloning and Nucleotide Sequencing of a TFPI-2 cDNA. A human placental cDNA library was screened with an oligonucleotide probe that could encode a protein homologous to Kunitz inhibitor domains and exhibited positive signals on a Northern blot of human placental mRNA under conditions of reduced hybridization stringency. Since the sizes of the placental mRNAs did not correspond to the known transcript sizes of any previously identified proteins containing Kunitz inhibitor domains, these data implied the existence of an additional protein or proteins in placenta with structures possibly related to novel Kunitz inhibitors. During the library screening, several plaques were identified that showed positive hybridization with the oligonucleotide probe at reduced hybridization stringency (60°C). Fourteen of the plaques were purified, 4 of which were related to each other

```
        10        20        30        40        50        60        70        80        90       100       110       120
GGACGCCTTGCCCAGCGGGCCGCCCGACCCCCTGCACCATGGACCCCGCTCGCCCCCTGGGGCTGTCGATTCTGCTGCTTTTCCTGACGGAGGCTGCACTGGGCGATGCTGCTCAGGAGCCAACA 125
                                  M  D  P  A  R  P  L  G  L  S  I  L  L  L  F  L  T  E  A  A  L  G  D  A  A  Q  E  P  T  29

  130       140       150       160       170       180       190       200       210       220       230       240       250
GGAAATAACGCGGAGATCTGTCTCCTGCCCCTAGACTACGGACCCTGCCGGGCCCTACTTCTCCGTTACTACTACGACAGGTACACGCAGAGCTGCCGCCAGTTCCTGTACGGGGGCTGCGAGGG 250
 G  N  N  A  E  I  C  L  L  P  L  D  Y  G  P  C  R  A  L  L  R  Y  Y  Y  D  R  Y  T  Q  S  C  R  Q  F  L  Y  G  G  C  E  G  71
          ********************************************************************************************************************

        260       270       280       290       300       310       320       330       340       350       360       370
CAACGCCAACAATTTCTACACCTGGGAGGCTTGCGACGATGCTTGCTGGAGGATAGAAAAAGTTCCCAAAGTTTGCCGGCTGCAAGTGAGTGTGGACGACCAGTGTGAGGGGTCCACAGAAAAGT 375
  N  A  N  N  F  Y  T  W  E  A  C  D  D  A  C  W  R  I  E  K  V  P  K  V  C  R  L  Q  V  S  V  D  D  Q  C  E  G  S  T  E  K  112
**********************************************                ***********************************************************

  380       390       400       410       420       430       440       450       460       470       480       490       500
ATTTCTTTAATCTAAGTTCCATGACATGTGAAAAATTCTTTTCCGGTGGGTGTCACCGGAACCGGATTGAGAACAGGTTTCCAGATGAAGCTACTTGTATGGGCTTCTGCGCACCAAAGAAAATT 500
Y  F  F  N  L  S  S  N  T  C  E  K  F  F  S  G  G  C  H  R  N  R  I  E  N  R  F  P  D  E  A  T  C  M  G  F  C  A  P  K  K  I  154
**************************************************************************************************************************

        510       520       530       540       550       560       570       580       590       600       610       620
CCATCATTTTGCTACAGTCCAAAAGATGAGGGACTGTGCTCTGCCAATGTGACTCGCTATTATTTTAATCCAAGATACAGAACCTGTGATGCTTTCACCTATACTGGCTGTGGAGGGAATGACAA 625
 P  S  F  C  Y  S  P  K  D  E  G  L  C  S  A  N  V  T  R  Y  Y  F  N  P  R  Y  R  T  C  D  A  F  T  Y  T  G  C  G  G  N  D  N  196
          ********************************************************************************************************************

  630       640       650       660       670       680       690       700       710       720       730       740       750
TAACTTTGTTAGCAGGGAGGATTGCAAACGTGCATGTGCAAAAGCTTTGAAAAAGAAAAAGAAGATGCCAAAGCTTCGCTTTGCCAGTAGAATCCGGAAAATTCGGAAGAAGCAATTTTAAACAT 750
 N  F  V  S  R  E  D  C  K  R  A  C  A  K  A  L  K  K  K  K  K  N  P  K  L  R  F  A  S  R  I  R  K  I  R  K  K  Q  F  *       235
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        760       770       780       790       800       810       820       830       840       850       860       870
TCTTAATATGTCATCTTGTTTGTCTTTATGGCTTATTTGCCTTTATGGTTGTATCTGAAGAATAATATGACAGCATGAGGAAACAAATCATTGGTGATTTATTCACCAGTTTTTATTAATACAAG 875

  880       890       900       910       920       930       940       950       960       970
TCACTTTTTCAAAAATTTGGATTTTTTTATATATAACTAGCTGCTATTCAAATGTGAGTCTACCATTTTTAATTTATGGTTCAACTGTTTGTGAGACTGAATTC                    979
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FIG. 1. Nucleotide sequence of cDNA clone for TFPI-2. The predicted signal peptide is underlined. The Kunitz-type domains are underscored with stars.

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TFPI-2          MDPARPLGLSILLLFLTEAALQ----------DAAQEPTGNNAEICLLPLDYGPCRALLLRYYYDRYTQSCRQFLYGGCEGNANNFYTWEACDDACW   87
                                    ^
TFPI    MIYTMKKVHALWASVCLLLNLAPAPLNADSEEDEEHTIITDTELPPLKLMHSFCAFKADDGPCKAIMKRFFFNIFTRQCEEFIYGGCEGNQNRFESLEECKKMCT  105
                                    ^

TFPI-2 R-----------IEKVPKVCRLQVSVDDQCEGSTEKYFFNLSSMTCEKFFSGGCHRNRIENRFPDEATCMGFCAPKKI-------------------------  154
TFPI    RDNANRIIKTTLQQEKPDFCFLEED-PGICRGYITRYFYNNQTKQCERFKYGGCLGN--MNNFETLEECKNICEDGPNGFQVDNYGTQLNAVNNSLTPQSTKVPS  207

TFPI-2 ------PSFCYSPKDEGLCSANVTRYYFNPRYRTCDAFTYTGCGGNDNNFVSREDCKRACAK-ALKKKKKMPKLRFASRIRKI-RKKQF              235
TFPI    LFEFHGPSWCLTPADRGLCRANENRFYYNSVIGKCRPFKYSGCGGNENNFTSKQECLRACKKGFIQRISKGG-LIKTKRKRKKQRVKIAYEEIFVKNM        304
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FIG. 2. Amino acid sequence alignment of TFPI-2 with TFPI. The last amino acid of each potential signal peptide is marked with a caret. The Kunitz-type domains are indicated by stars. TFPI-2 sequence identities with TFPI are indicated by vertical lines, and conservative amino acid substitutions are indicated by colons.

by DNA hybridization. These four plaques coded for a homolog to the amyloid precursor protein, APPH (12). The remaining 10 clones were also related to each other by hybridization analysis but distinct from APPH. The longest clone contained a cDNA insert of ≈990 bp and appeared to represent a full-length transcript with a 5′ noncoding region of 38 nt, an open reading frame of 705 nt encoding a 235-aa protein, and a 3′ noncoding region of 235 nt.

The encoded protein shows considerable sequence and overall domain organization similarity with human tissue factor pathway inhibitor (Fig. 1). In view of the structural similarities between this protein and TFPI, we refer to this protein as TFPI-2. Like TFPI, TFPI-2 appears to have a classical signal peptide that directs insertion into the endoplasmic reticulum and secretory pathway, a short amino-terminal region rich in acidic amino acids, three tandem Kunitz-type inhibitor domains, and a short carboxyl-terminal domain enriched in basic amino acids. The signal peptide is predicted to be cleaved following Gly-22, which conforms to the −1, −3 rules of signal peptidase specificity (19). Following the signal peptide is a short region of 13 aa in which the only charged residues are three glutamic residues. The acidic region is followed by three tandem Kunitz-type inhibitor domains that are homologous to the three tandem Kunitz-type domains in TFPI (Fig. 2). The predicted secondary folding structure for TFPI-2 is illustrated in Fig. 3. Individually, the Kunitz-type domains of TFPI-2 are homologous to the corresponding domains in TFPI as follows. Domain 3 is the most highly conserved, with 53% sequence identity with domain 3 of TFPI. Domain 1 exhibits 43% identity and domain 2 exhibits 35% sequence identity. The Kunitz-type domain 2 in TFPI-2 is also unusual in that it contains an additional 2 aa between the fourth and fifth cysteine residues, making this loop longer than most Kunitz-type family members. The presumed P_1 reactive-site residue of the first Kunitz-type domain in TFPI-2 is arginine, whereas lysine

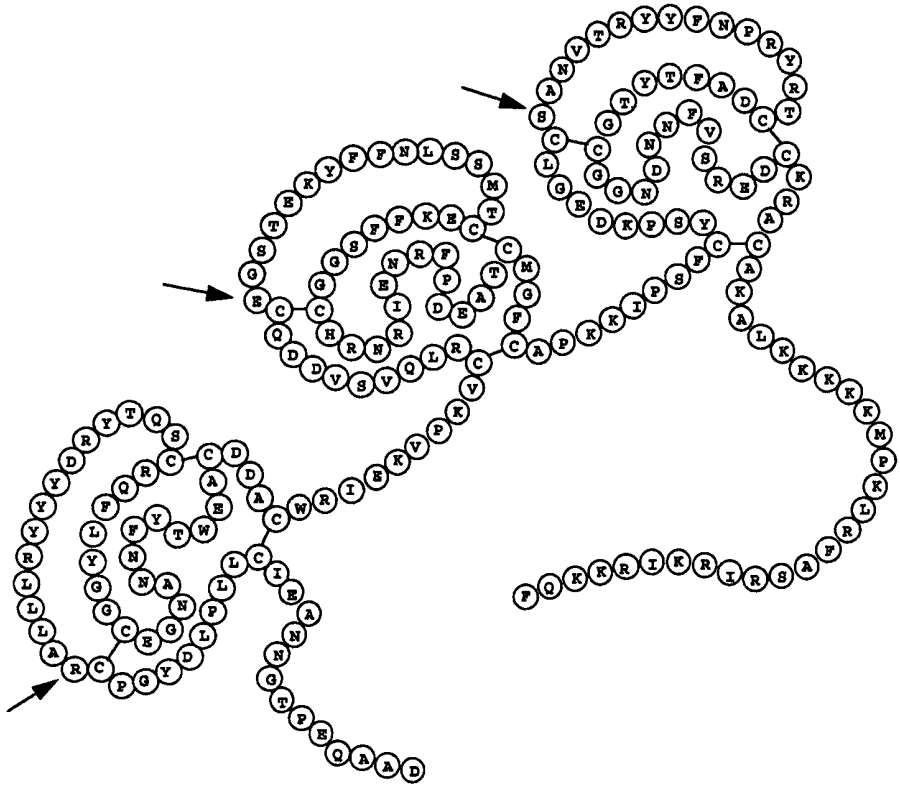

FIG. 3. Predicted secondary folding structure for TFPI-2. Disulfide bonds are assumed on the basis of the crystal structure of bovine pancreatic trypsin inhibitor (20). Arrows indicate the locations of the presumed P_1 residues of the reactive-site cleft for the Kunitz domains.

occupies this position in TFPI. The presumed P_1 position in the second domain of TFPI-2 is glutamic acid, while arginine is found in this position in TFPI. The presumed P_1 position of the third Kunitz-type domain in TFPI-2 is serine. The sequence alignment of TFPI-2 with TFPI indicates that the spacer sequences between individual Kunitz-type domains in TFPI-2 are considerably shorter than those observed in TFPI.

Following the Kunitz-type domains in TFPI-2 is a carboxyl-terminal tail of 27 aa of which ≈50% (13/27) are basic, including a stretch of 5 consecutive lysine residues. This region in TFPI-2 is highly homologous to the corresponding region in TFPI, which is slightly longer but also contains 13 basic amino acids that include a consecutive stretch of 6 basic residues. In TFPI, this region is highly susceptible to proteolysis and is essential for full anticoagulant activity in dilute thromboplastin clotting-time assays (9).

The cDNA sequence also encodes two putative N-linked glycosylation sites in TFPI-2, at Asn-116 and Asn-170 in the second and third Kunitz-type domains, respectively. Based on a calculated molecular mass of 24.6 kDa for the carbohydrate-free protein and an observed molecular mass of 32 kDa for the expressed protein (see below), it is very likely that both Asn-116 and Asn-170 are glycosylated in the secreted molecule.

Northern blot analysis of human tissues with TFPI-2 cDNA reveals that this gene is transcribed in human umbilical vein endothelial cells, placenta, and liver (data not shown). One major transcript is apparent at 1.4 kb with a possible minor transcript at ≈2 kb. From the size of our longest clones, we are apparently reporting an incomplete transcript that is missing some of the 3′ noncoding sequence, since no polyadenylylation sequence is seen. The *Eco*RI site at the 3′ end appears to be an internal site, as no linker sequence is seen at this end. Therefore, the mRNA size would predict an additional 400 bp of 3′ (or 5′) noncoding sequence in a full-length transcript.

Expression, Purification, and Characterization of Recombinant TFPI-2. The cDNA for TFPI-2 was inserted into a mammalian cell expression vector and expressed in BHK cells as described above. Conditioned medium from clonal cell lines was assayed for trypsin-inhibitory activity and one clone exhibiting the highest level of trypsin-inhibitory activity was selected for preparative-scale culture. Recombinant TFPI-2 was purified to homogeneity from ≈5 liters of BHK serum-free conditioned medium by a combination of heparin-agarose chromatography, ion-exchange FPLC, and Superose 12 chromatography. In this procedure, aprotinin added to the medium to prevent degradation of the TFPI-2 was effectively separated from recombinant TFPI-2 in the 0.2 M NaCl wash of the heparin-agarose column. Approximately 1 mg of purified TFPI-2 was obtained from 5 liters of conditioned medium by this procedure. Purified recombinant TFPI-2 migrated as a single band in SDS/PAGE with an apparent molecular mass of 32 kDa in the presence or absence of 10% 2-mercaptoethanol (Fig. 4). Amino-terminal sequence analysis of a single preparation of recombinant TFPI-2 indicated a major sequence (≈70%) of Asp-Ala-Ala-Gln-Glu-Pro-Thr-Gly-Asn-Asn and a minor sequence (≈30%) of Ala-Gln-Glu-Pro-Thr-Gly-Asn-Asn, suggesting either alternative cleavage sites for the signal peptidase or possible amino-terminal degradation by exopeptidases during TFPI-2 purification. In spite of its structural homology to TFPI, the purified recombinant TFPI-2 failed to react with rabbit polyclonal anti-human TFPI IgG in a sensitive ELISA (data not shown).

As purified recombinant TFPI-2 strongly inhibited the amidolytic activity of trypsin, we next determined the effect of purified recombinant TFPI-2 on the amidolytic activities of human factor Xa, thrombin, and the factor VIIa–tissue factor complex. Recombinant TFPI-2 inhibited the amidolytic activity of 10 nM factor VIIa–tissue factor toward the chromogenic substrate S-2288 in a dose-dependent manner (Fig. 5). The presence of 100 nM S376A factor X enhanced this reaction, suggesting complex formation between nascent S376A factor Xa and TFPI-2 that modulated the inhibition of factor VIIa–tissue factor by TFPI-2 similar to, but not to the same extent as, that observed for TFPI (7). Further, in the presence of heparin at 0.2 unit/ml, the concentration of TFPI-2 required to half-maximally inhibit 10 nM factor VIIa–tissue factor was reduced ≈10-fold relative to the absence of heparin (Fig. 5). Control studies indicated that heparin at 0.2 unit/ml had no effect on factor VIIa–tissue factor amidolytic activity. Under comparable conditions, TFPI-2 at high concentrations weakly inhibited the amidolytic activity of 10 nM

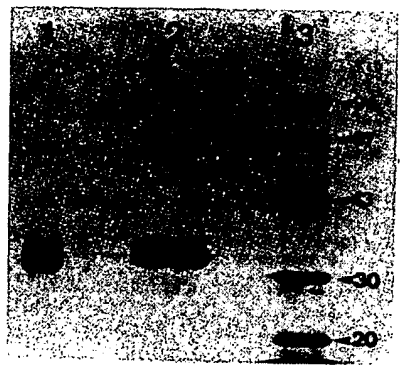

FIG. 4. SDS/PAGE of recombinant TFPI-2. Reduced (with 10% 2-mercaptoethanol) and unreduced samples were electrophoresed in a 10% polyacrylamide slab gel. Lane 1, 15 μg of unreduced TFPI-2; lane 2, 15 μg of reduced TFPI-2; lane 3, mixture of reduced standard proteins including phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), and soybean trypsin inhibitor (20 kDa).

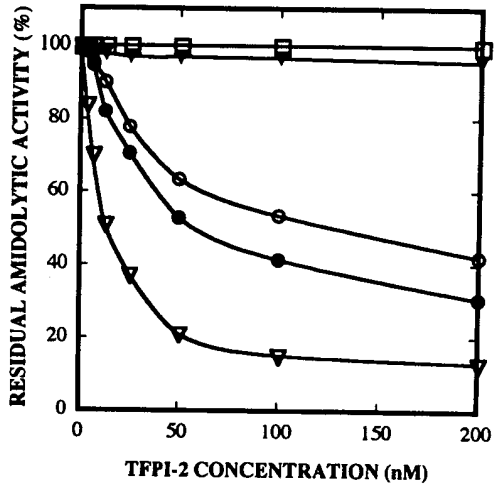

FIG. 5. Effect of recombinant TFPI-2 on the amidolytic activities of human thrombin, human factor Xa, and a complex of human factor VIIa–tissue factor. Various concentrations of TFPI-2 were incubated at 37°C for 15 min with either 10 nM thrombin (□), 10 nM factor Xa (▼), or 10 nM factor VIIa–tissue factor (○) in a microtitration plate. In separate experiments, various concentrations of TFPI-2, in the presence of either 100 nM S376A factor X (●) or heparin at 0.2 unit/ml (▽) were incubated with 10 nM factor VIIa–tissue factor. After a 15-min incubation, 50 μl of either 3 mM S-2238 (□), 3 mM S-2222 (▼), or 3 mM S-2288 (○, ●, ▽) was added to the well and A_{405} was determined in a kinetic plate reader. Each point represents the average of triplicate determinations.

human factor Xa toward S-2222 but was without effect on the amidolytic activity of human thrombin toward S-2238 (Fig. 5). In contrast to the results observed for the inhibition of factor VIIa–tissue factor by recombinant TFPI-2, the weak inhibition of factor Xa amidolytic activity by recombinant TFPI-2 was not measurably affected by the presence of heparin at 0.2 unit/ml.

DISCUSSION

The present data establish the existence of a Kunitz-type tissue factor-pathway inhibitor, TFPI-2, that is structurally related to human plasma TFPI. A full-length cDNA for TFPI-2 was isolated from a human placenta cDNA library, and plasmids containing this cDNA were constructed and transfected into BHK cells. Recombinant TFPI-2 was purified from BHK cell conditioned medium and migrated in SDS/PAGE as a single band at 32 kDa. Purified recombinant TFPI-2 exhibited an amino-terminal sequence identical to that predicted by the cDNA, although ≈30% of the preparation lacked a dipeptide at the amino terminus, suggesting either an alternative cleavage site for the signal peptidase or amino-terminal processing by an exopeptidase during TFPI-2 purification. Recombinant TFPI-2 was a relatively strong inhibitor of factor VIIa–tissue factor amidolytic activity but inhibited only weakly the amidolytic activity of factor Xa. Heparin augmented the inhibitory activity of TFPI-2 toward the factor VIIa–tissue factor complex, presumably through its interaction with the polycationic carboxyl-terminal tail of TFPI-2.

The striking degree of amino acid sequence homology and the conservation of overall domain structure between TFPI and TFPI-2 indicate that these genes have, in all likelihood, evolved by gene duplication. The highly conserved regions of sequence within Kunitz-type inhibitor domains, as well as the carboxyl-terminal basic amino acid-rich region, strongly suggests functional similarity between these proteins within their relative biological contexts. While studies (6–8) have shown that TFPI alone is capable of inhibiting factor VIIa–tissue factor activity, it is a relatively weak factor VIIa–tissue factor inhibitor in the absence of factor Xa (7). Although not proven, it is assumed that TFPI inhibits factor VIIa–tissue factor in the absence of factor Xa through the interaction of the factor VIIa active site and the P_1 lysine residue of the first Kunitz-type domain. In contrast to TFPI, TFPI-2 has an arginine in the P_1 position in the first Kunitz-type domain, whereas the P_1 residues in the second and third Kunitz-type domains are glutamic acid and serine, respectively. Thus, the second Kunitz-type domain in TFPI-2 may have lost its specificity for factor Xa. In addition, given that factor VIIa–tissue factor exhibits a kinetic preference for synthetic substrates with a P_1 arginine residue (21), it is very likely that the first Kunitz-type domain in TFPI-2 is responsible for the inhibition of factor VIIa–tissue factor amidolytic activity observed in this study.

While the physiological function of TFPI-2 is unknown, the localization of TFPI-2 in umbilical vein endothelial cells, placenta, and liver, coupled with its ability to inhibit factor VIIa–tissue factor activity, suggests a specialized role for this protein in hemostasis. The placenta is rich in tissue factor (22) and is a highly vascularized tissue with high volume blood flow to promote efficient nutrient exchange between the maternal and fetal vascular systems. Inasmuch as Northern analyses revealed 100–1000 times more TFPI-2 mRNA than TFPI mRNA in a poly(A)$^+$ RNA preparation from placental tissue (C.A.S. and D.C.F., unpublished results), it is tempting to speculate that TFPI-2 plays a role in the regulation of tissue factor-initiated coagulation in placental tissue and may be important for hemostasis in pregnancy. In this connection, it is particularly noteworthy that pulmonary embolism represents the leading non-obstetric cause of postpartum death (23). Accordingly, it will be of great interest to determine whether TFPI-2 circulates in blood and, if so, whether its plasma level changes in women during and after pregnancy.

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